Ocena ekspresji wybranych czynników immunomodulujących w raku płaskonabłonkowym jamy ustnej

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SPIS TREŚCI

OBJAŚNIENIA SKRÓTÓW I SYMBOLI UŻYWANYCH W PRACY

ANOVA	*Analysis of variance*	Jednoczynnikowa analiza wariancji
APC	*Antigen-presenting cell*	Komórka prezentująca antygen
B7	*B7 protein family*	Rodzina białek B7
B7-1/CD80/B7/ B7.1/BB1/ CD28LG/CD28L G1/LAB7	*B7-1 protein/Cluster of differentiation 80*	Białko B7-1/Kompleks różnicowania 80
B7-2/CD86/B7.2/ B70/ CD28LG2/ LAB72	*B7-2 protein/Cluster of differentiation 86*	Białko B7-2/Kompleks różnicowania 86
B7-DC/CD273 /PD-L2/B7DC/ PDCD1LG2/ PDL2/Btdc/ PDCD1L2/ bA574F11.2	*B7-DC protein/Cluster of differentiation 273/ Programmed death ligand 2*	Białko B7-DC/Kompleks różnicowania 273/ Ligand programowanej śmierci 2
B7-H1/CD274/ PD-L1	*B7-H1 protein/Cluster of differentiation 274/ Programmed death ligand 1*	Białko B7-H1/Kompleks różnicowania 274/ Ligand programowanej śmierci 1
B7-H2/CD275/ ICOSL/B7h/ B7RP-1/ LICOS	*B7-H2 protein/Cluster of differentiation 275*	Białko B7-H2/Kompleks różnicowania 275
B7-H3/CD276/ CD276/ 4Ig-B7-H3/B7H3/ B7RP-2	*B7-H3 protein/Cluster of differentiation 276*	Białko B7-H3/Kompleks różnicowania 276
B7-H4/B7x/ B7S1/Vtcn1	*B7-H4 protein*	Białko B7-H4
B7-H5/VISTA /Vsir/PD-1H/ DD1a/Dies1/ Gi24/SISP1/ C10orf54	*B7-H5 protein/V-domain Ig suppressor of T cell activation/ V-set immunoregulatory receptor*	Białko B7-H5/Supresor V w domenie V/receptor immunoregulacyjny V-set
B7-H6	*B7-H6 protein*	Białko B7-H6
B7-H7	*B7-H7 protein*	Białko B7-H7

Bcl-xL	*Apoptosis regulator protein*	Białko regulujące apoptozę
CD28	*Cluster of differentiation 28*	Kompleks różnicowania 28
CD28H/ TMIGD2/ IGPR1	*Transmembrane and immunoglobulin domain containing 2*	Domena transbłonowa i immunoglobulinowa 2
CD80/B7-1	*Cluster of differentiation 80/B7-1 protein*	Kompleks różnicowania 80/ Białko B7-1
CD86/B7-2	*Cluster of differentiation 80/B7-2 protein*	Kompleks różnicowania 86/ Białko B7-2
CD152/CTLA-4	*Cluster of differentiation 152/ Cytotoxic T-lymphocyte-associated protein 4*	Kompleks różnicowania 152/ Antygen-4 związany z limfocytem
CD273/B7-DC/ PD-L2	*B7-DC protein/Cluster of differentiation 273/ Programmed death ligand 2*	Białko B7-DC/Kompleks różnicowania 273/ Ligand programowanej śmierci 2
CD274/B7-H1/ PD-L1	*B7-H1 protein/Cluster of differentiation 274/ Programmed death ligand 1*	Białko B7-H1/Kompleks różnicowania 274/Ligand programowanej śmierci 1
CD275/B7-H2/ ICOSL/B7h/ B7RP-1/ LICOS	*Cluster of differentiation 275/ B7-H2 protein*	Kompleks różnicowania 275/ Białko B7-H2
CD276/B7-H3	*B7-H2 protein/Cluster of differentiation 276*	Białko B7-H2/Kompleks różnicowania 276
CD278/ICOS	*Cluster of differentiation 278/ Inductible T-cell costimulator*	Kompleks różnicowania 278/ indukcyjny ko stymulator komórek T
CD279/PD-1	*Cluster of differentiation 279/ Programmed death receptor 1*	Kompleks różnicowania 279/ Receptor programowanej śmierci 1
CTLA-4/CD152	*Cytotoxic T-lymphocyte-associated protein 4/Cluster of differentiation* CD152)	Antygen-4 związany z limfocytem T/Kompleks różnicowania 152
HIER	*Heat induced epitope retrieval*	Metoda wysokotemperaturowego odsłaniania epitopów
HPV	*Human Papilloma Virus*	Wirus brodawczaka ludzkiego

ICOS/CD278	*Cluster of differentiation 278/ Inductible T-cell costimulator*	Kompleks różnicowania 278/ Indukcyjny ko-stymulator komórek T
IFN-γ	*Interferone γ*	Interferon γ
IgC	*Immunoglobulin domain C*	Immunoglobulinowa domena C
IgV	*Immunoglobulin domain V*	Immunoglobulinowa domena V
IHC	*Immunohistochemistry*	Immunohistochemia
IL-1α	*Interleukin IL-1α*	Interleukina IL-1α
IL-1β	*Interleukin IL-1β*	Interleukina IL-1β
IL-1RA	*Interleukin IL-1RA*	Interleukina IL-1RA
IL-2	*Interleukin 2*	Interleukina 2
ILC2	*Innate lymphoid cells 2*	Wrodzone komórki limfoidalne 2
IL-4	*Interleukin 4*	Interleukina 4
IL-5	*Interleukin 5*	Interleukina 5
IL-6	*Interleukin 6*	Interleukina 6
IL-10	*Interleukin 10*	Interleukina 10
IL-13	*Interleukin 13*	Interleukina 13
IL-18	*Interleukin* IL-18	Interleukina IL-18
IL-33	*Interleukin 33*	Interleukina 33
IL-36α	*Interleukin IL-36α*	Interleukina IL-36α
IL-36β	*Interleukin IL-36β*	Interleukina IL-36β
IL-36Ra	*Interleukin IL-36Ra*	Interleukina IL-36Ra
IL-37	*Interleukin IL-37*	Interleukina IL-37
IL-38	*Interleukin IL-38*	Interleukina IL-38
ITIM	*Immunoreceptor tyrosine-bases inhibitory motif*	Immunoreptorowy motyw hamujący na bazie tyrozyny
ITSM	*Immunoreceptor tyrosine-based switch motif*	Immunoreptorowy motyw przełącznika na bazie tyrozyny
LCS	*Liquid coverslip*	Ciecz nakrywkowa

NK/NK cells	*Natural killers*	Limfocyty „naturalni zabójcy”
NKp30	*Receptor NKp30*	Receptor NKp30
M	*Metastasis*	Przerzuty odległe narządowe
MHC	*Major histocompatibility complex*	Główny układ zgodności tkankowej
N	*Nodules*	Przerzuty w węzłach chłonnych,
NE	*No examined*	Nie badano
OS	*Overall survival*	Całkowite przeżycie
OSCC/RJU	*Oral squamous cell carcinoma*	Rak płaskonabłonkowy jamy ustnej
p	*p value*	Poziom istotności
PD-1/CD279	*Programmed death receptor 1/ Cluster of differentiation 279*	Receptor programowanej śmierci/ Kompleks różnicowania 279
PD-L1/ B7-H1/ CD274/	*Programmed death ligand 1/ B7-H1 protein/Cluster of differentiation 274*	Ligand programowanej śmierci 1/Białko B7-H1/Kompleks różnicowania 274
PD-L2/B7-DC/ CD273	*Programmed death ligand 2/ B7-DC protein/Cluster of differentiation 273*	Ligand programowanej śmierci 2/Białko B7-DC/Kompleks różnicowania 273
PBS	*Phosphate buffered saline*	Bufor fosforanowy
PI-3K	*Phosphatidyl-inositol 3-kinase*	Wewnątrzkomórkowa ścieżka sygnałowa kinazy 3-fosfatydyloinozytolu
RGMB	*Repulsive Guidance Molecule BMP Co-Receptor B*	Ko-receptor odpychający cząsteczkę BMP
RJU/OSCC	*Oral squamous cell carcinoma*	Rak płaskonabłonkowy jamy ustnej
SHP1	*Src homology region 2 domain-containing phosphatase-1*	Fosfataza 1 homologiczna z 2 regionem Src

SHP2	*Src homology region 2 domain-containing phosphatase-2*	Fosfataza 2 homologiczna z 2 regionem Src
ST2	*Membrane receptor soluble interleukin 1 receptor-like 1*	Receptor błonowy ST2
T	*Tumour*	Wielkość guza pierwotnego
TCR	*T-cell receptor*	Receptor komórek T
TCs	*Tumour cells*	Komórki nowotworowe
TILs	*Tumour infiltrating lymphocytes*	Limfocyty towarzyszące nowotworowi
TMA	*Tissue microarray*	Mikromacierze tkanokowe
TMIGD2/ IGPR1 /CD28H	*Transmembrane and immunoglobulin domain containing 2*	Domena transbłonowa i immunoglobulinowa 2
TNF	*Tumour necrosis factor*	Czynnik martwicy nowotworów
TNM	*Classification of Malignant Tumours (Tumour, nodules, metastasi)*	Klasyfikacja guzów złośliwych (Guz, przerzuty do węzłów chłonnych, przerzuty odległe)
WHO	*World Health Organisation*	Światowa Organizacja Zdrowia
VISTA/B7-H5/ Vsir/PD-1H/DD1a /Dies1/Gi24/SISP1 /C10orf54	*B7-H5 protein/V-domain Ig suppressor of T cell activation/ V-set immunoregulatory receptor*	Białko B7-H5/Supresor V w domenie V/receptor immunoregulacyjny V-set
Vsir/B7-H5/ VISTA/PD-1H/ DD1a/Dies1/ Gi24/SISP1/ C10orf54	*B7-H5 protein/V-domain Ig suppressor of T cell activation/ V-set immunoregulatory receptor*	Białko B7-H5/Supresor V w domenie V/receptor immunoregulacyjny V-set
ZAP70	*Zetachain-associated protein kinase 70*	Kinaza białkowa 70 łańcucha zeta

1. Wprowadzenie

Rozprawa doktorska:

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powstała w oparciu o publikacje:

1 **Adamski ŁJ,** Starzyńska A, Adamska P, Kunc M, Sakowicz-Burkiewicz M, Marvaso G, Alterio D, Korwat A, Jereczek-Fossa BA, Rafał Pęksa R. High PD-L1 expression on tumor cells indicates worse overall survival in advanced oral squamous cell carcinomas of the tongue and the floor of the mouth but not in other oral compartments. *Biomedicines* 2021, 9, 1132. **IF: 6,081; MNiSW: 20** https://doi.org/10.3390/biomedicines9091132 [https://www.mdpi.com/2227-9059/9/9/1132]

2 Starzyńska A, Sejda A, **Adamski ŁJ**, Adamska P, Pęksa R, Sakowicz-Burkiewicz M, Wychowański P, Jereczek-Fossa BJ. The B7 family molecules in oral squamous cell carcinoma: a systematic review. PART I: B7-H1 (PD-L1) and B7-DC (PD-L2). *Adv Dermatol Allergol* 2020; doi.org/10.5114/ada.2020.98522. **IF: 1,837; MNiSW: 70;** [https://www.termedia.pl/The-B7-family-molecules-in-oral-squamous-cell-carcinoma-a-systematic-review-Part-I-B7-H1-PD-L1-and-B7-DC-PD-L2-,7,41686,0,1.html]

3 Starzyńska A, Sejda A, **Adamski ŁJ**, Adamska P, Pęksa R, Sakowicz-Burkiewicz M, Wychowański P, Jereczek-Fossa BJ. The B7 family molecules in oral squamous cell carcinoma: a systematic review.PART II: B7-1, B7-2, B7-H2, B7-H3, B7-H4, B7-H5 (VISTA), B7-H6 and B7-H7. *Adv Dermatol Allergol* 2020; doi.org/10.5114/ada.2020.98523. **IF: 1,837; MNiSW: 70 pkt** [https://www.termedia.pl/The-B7-family-molecules-in-oral-squamous-cell-carcinoma-a-systematic-review-Part-II-B7-1-B7-2-B7-H2-B7-H3-B7-H4-B7-H5-VISTA-B7-H6-and-B7-H7,7,41687,0,1.html]

oraz niniejszą dysertację.

2. STRESZCZENIE

W prawidłowo funkcjonującym organizmie uszkodzone komórki podlegają apoptozie, w której uczestniczą elementy układu odpornościowego. W patologii nowotworów komórki zyskują nieograniczoną zdolność podziałów i przeżycia poprzez zmiany w materiale genetycznym. Receptory należące do rodziny CD28 znajdują się na komórkach układu odpornościowego. Ligandy (rodzina B7) obecne są w na komórkach prezentujących antygen, komórkach nabłonka, osteoblastach, fibroblastach oraz na komórkach zmienionych nowotworowo. Szlak B7-CD28 ma możliwość regulowania odpowiedzi immunologicznej poprzez ograniczenie czasu i siły reakcji zapalnej. Natomiast IL-33 jest cytokiną prozapalną, która jest głównie związana z gojeniem się ran i urazami.

Celem pracy była ocena ekspresji białek PD-L1 i IL-33 u pacjentów z rakiem płaskonabłonkowym jamy ustnej w odniesieniu do cech klinicznych i przeżycia pacjentów.

Badaną grupę stanowiło 95 chorych leczonych z powodu raka płaskonabłonkowego jamy ustnej w Uniwersyteckim Centrum Klinicznym w Gdańsku w latach 2007-2012. W badaniu zostały wykorzystane preparaty histopatologiczne z pierwotnych guzów raka płaskonabłonkowego jamy ustnej.

Badania immunohistochemiczne zostały przeprowadzone na mikromacierzach tkankowych. Białka oceniano oddzielnie na komórkach nowotworowych (ang. *tumor cells,* TCs) oraz limfocytach naciekających guz (ang. *tumor infiltrating lymphocytes,* TILs).

Wysoka ekspresja białka PD-L1 w obrębie TILs wiązała się z lepszym całkowitym przeżyciem w analizie jednoczynnikowej. Guzy zlokalizowane w dnie jamy ustnej i w obrębie języka miały niższy odsetek PD-L1-dodatnich TCs w porównaniu z innymi lokalizacjami. Wysoka ekspresja PD-L1 w TCs nie miała znaczenia prognostycznego, gdy analizowano całą kohortę. Ekspresja PD-L1 wraz z rozmiarem zmiany pierwotnej w modelu wieloczynnikowym była związana z wyższym ryzykiem zgonu w przypadku raka dna jamy ustnej i języka (HR = 2,51; 95% CI = 1,97-5,28). Przerzuty do węzłów chłonnych były czynnikiem prognostycznym w pozostałych

lokalizacjach w analizie wieloczynnikowej (HR 0,24; 95% CI 0,08-0,70). Ekspresja IL-33 nie miała wpływu na całkowite przeżycie chorych.

Znaczenie prognostyczne PD-L1 w raku jamy ustnej zależy od lokalizacji i typu komórki wyrażającej receptor immunologicznego punktu kontrolnego (TCs *vs.* TILs).

3. SUMMARY

In a properly functioning organism, damaged cells undergo apoptosis, a process in which various elements of the immune system participate. In tumour pathology, cells gain unlimited ability to divide and survive as a result of changes in their genetic material. Receptors belonging to the CD28 family are found on cells of the immune system. Ligands (family B7) are present on antigen presenting cells, epithelial cells, osteoblasts, fibroblasts and neoplastic cells. The B7-CD28 pathway has the ability to regulate the immune response by limiting the timing and strength of the inflammatory response. IL-33 is mostly connected with wounds and injuries such as proinflammation cytokine.

The aim of the study was to evaluate PD-L1 and IL-33 expression in patients with OSCC in relation to clinical characteristics and survival.

The study group included 95 patients treated for oral squamous cell carcinoma at the University Clinical Centre in Gdańsk between 2007-2012. Study was performed on histopathological samples from primary OSCC.

Immunohistochemical and molecular tests were carried out on tissue microarrays. PD-L1 and IL-33 were assessed separately in tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs).

High PD-L1 expression in TILs was associated with better overall survival (OS) in univariate analysis. Tumors localized in the floor of the oral cavity and tongue tended to have a lower percentage of PD-L1-positive TCs when compared to other locations. PD-L1 expression on TCs had no prognostic significance when the whole cohort was analyzed. However, along with the T descriptor, it was included in the multivariable model predicting death in carcinomas of the floor of the oral cavity and tongue (HR = 2.51, 95% CI = 1.97-5.28). In other locations, only nodal status was identified as an independent prognostic factor in multivariate analysis (HR = 0.24, 95% CI = 0.08-0.70). Expression of IL-33 had no impact on survival, but it was differently expressed in various locations.

This study suggests that PD-L1 and IL-33 proteins can be potential prognostic factors in oral squamous cell carcinoma.

4. WSTĘP

Dziewięćdziesiąt pięć procent nowotworów złośliwych jamy ustnej stanowi rak płaskonabłonkowy (ang. *oral squamous cell carcinoma,* OSCC). Zmiany dotyczą głównie mężczyzn po 60 roku życia [1]. Wśród czynników, które wypływają na większe ryzyko wystąpienia raka jamy ustnej wymienia się palenie tytoniu, żucie betelu, spożywanie wysokoprocentowego alkoholu czy infekcje wirusem brodawczaka ludzkiego (ang. *Human Papilloma Virus,* HPV) [2-7]. Owrzodzenie błony śluzowej jest najczęstszym objawem [2,8,9].

Klasyfikacja TNM (T – *tumour* – wielkość guza pierwotnego, N – *nodules* – przerzuty w węzłach chłonnych, M – *metastasis* – przerzuty odległe narządowe) została opracowana w celu ujednolicenia oceny stopnia zaawansowania klinicznego i patomorfologicznego nowotworów złośliwych. W niniejszej pracy użyto klasyfikacji z 2016 roku (Tabela 1 i 2). Ósmą klasyfikację rozszerzono o ocenę stopienia naciekania miejscowego tkanek przez nowotwór oraz obecności nacieku torebki węzłów chłonnych [10].

Pomimo znacznego postępu, jaki poczyniono w leczeniu raka płaskonabłonkowego jamy ustnej, przeżycie całkowite (ang. *overall survival,* OS) nie uległo znaczącej poprawie w ciągu ostatnich kilkudziesięciu lat. Leczeniem z wyboru jest zwykle zabieg chirurgiczny z następową radioterapią. Przebieg kliniczny choroby zależy głównie od stopnia zaawansowania zmiany, lokalizacji guza oraz możliwości radykalnej resekcji chirurgicznej. Raki zlokalizowane w obrębie języka i dna jamy ustnej charakteryzują się w momencie rozpoznania znacznym zaawansowaniem klinicznym oraz gorszym rokowaniem niż nowotwory w pozostałych lokalizacjach jamy ustnej. Mikrośrodowisko immunologiczne nowotworu może również wpływać na rokowanie lub celem spersonalizowanej terapii [11-13].

Tabela 1. Stopnień zaawansowania patomorfologicznego oraz klinicznego wg ósmej edycji klasyfikacji TNM (WHO 2016).

	Guz pierwotny
Tx	Nie można ocenić guza pierwotnego
T0	Brak dowodów świadczących o występowaniu pierwotnego nowotworu
Tis	Rak in situ
T1	Guz wielkości 2 cm lub mniejszy w największym wymiarze oraz **głębokość naciekania 5 mm lub mniejsza**
T2a	Guz wielkości 2 cm lub mniejszy w największym wymiarze oraz **głębokość naciekania większa niż 5 mm, ale mniejsza niż 10 mm**
T2b	Guz wielkości 2 cm, ale nie większy niż 4 cm w największym wymiarze oraz **głębokość naciekania nie większa niż 10 mm**
T3	Guz wielkości ponad 4cm w największym wymiarze lub **głębokość naciekania większa niż 10 mm**
T4a	Guz nacieka kość korową żuchwy/zatoki szczękowej/skórę twarzy
T4b	Guz nacieka przestrzeń żwacza/wyrostek skrzydłowaty/podstawę czaszki/zamyka wewnętrzną tętnicę szyjną
	Regionalne węzły chłonne
Nx	Regionalne węzły chłonne nie mogą być oceniane
N0	Brak przerzutów do regionalnych węzłów chłonnych
N1	Przerzuty w jednym węźle chłonnym po stronie guza, o wymiarze 3 cm lub mniejszym w największym wymiarze **bez nacieku pozawęzłowego**
N2a	Przerzuty w jednym węźle chłonnym po stronie guza, o wymiarze większym niż 3 cm, ale mniejszym niż 6 cm w największym wymiarze **bez nacieku pozawęzłowego**
N2b	Przerzuty w licznych węzłach chłonnych po stronie guza, o wymiarze nie większym niż 6 cm w największym wymiarze **bez nacieku pozawęzłowego**
N2c	Przerzuty w węzłach chłonnych obustronne lub po stronie przeciwnej do guza, o wymiarze nie większym niż 6 cm w największym wymiarze **bez nacieku pozawęzłowego**
N3a	Przerzuty w węzłach chłonnych większe niż 6 cm w największym wymiarze **bez nacieku pozawęzłowego**
N3b	Przerzuty do jednego lub licznych węzłów chłonnych **z naciekiem pozawęzłowym**
	Przerzuty odległe
M0	Brak przerzutów odległych
M1	Przerzuty odległe

Tabela 2. Stopnie zaawansowania klinicznego wg WHO 2016.

Stadium	T	N	M
I	T1	N0	M0
II	T2a, T2b	N0	M0
III	T3	N0	M0
	T1, T2a, T2b, T3	N1	M0
IVA	T4a	N0, N1	M0
	T1, T2a, T2b, T3, T4a	N2	M0
IVB	Każde T	N3	M0
	T4b	Każde N	M0
IVC	Każde T	Każde N	M1

4.1 Rodzina białek B7

W prawidłowo funkcjonującym organizmie uszkodzone komórki podlegają apoptozie, w której uczestniczą elementy układu odpornościowego. Główną rolę w tym procesie odgrywają limfocyty T i NK (ang. *natural killers*). W patologii nowotworów komórki zyskują nieograniczoną zdolność podziałów i przeżycia poprzez zmiany w materiale genetycznym. Aktywacja i funkcje limfocytów są rezultatem równowagi pomiędzy regulacją w górę (pobudzającą) i w dół (hamującą). Niektóre z nowotworów wykazują pewien stopień immunogenności. Występuje szereg mechanizmów molekularnych zaangażowanych w regulacje mikrośrodowiska komórek nowotworowych (ang. *tumor microenviroment*, TME) i chroniących przed atakiem układu odpornościowego [14]. Aktywacja komórek T wymaga dwóch sygnałów. Pierwszy z nich to aktywacja za pośrednictwem receptora komórek T (ang. *T-cell receptor,* TCR) – główny układ zgodności tkankowej (ang. *major histocompatibility complex,* MHC) na komórkach prezentujących antygen (ang. *antigen-presenting cell,* APC). Drugi sygnał opiera się na cząsteczkach kostymulujących, takich jak szlak B7-CD28 [15].

Receptory należące do rodziny CD28 znajdują się na komórkach układu odpornościowego. Głównie dotyczą limfocytów T towarzyszących nowotworowi (ang. *tumor infiltrating lymphocytes*, TILs). Ligandy (rodzina B7) obecne są w warunkach

fizjologicznych na komórkach APC, komórkach układu odpornościowego, komórkach nabłonka, osteoblastach, fibroblastach i innych. Dodatkowo wykryto obecność na komórkach zmienionych nowotworowo [16,17].

Szlak B7-CD28 odpowiada za możliwość regulowania odpowiedzi immunologicznej poprzez ograniczenie czasu i siły reakcji zapalnej. Mimo że nie jest znany dokładny mechanizm ko-stymulacji przez szlak B7-CD28, obecnie wykorzystywane są terapie ukierunkowane molekularnie w leczeniu zmian nowotworowych, chorób z autoagresji oraz zakaźnych [15,18]

Do rodziny białek B7 dotychczas zaliczono białka: B7-1 (CD80), B7-2 (CD86), B7-H2 (CD275, ICOSL, B7h, B7RP-1, LICOS), PD-L1 (CD274; B7-H1), PD-L2 (CD273; B7-DC), B7-H3 (CD276), B7-H4 (B7x, B7S1, Vtcn1), VISTA (PD-1H, Vsir, DD1a,Dies1, Gi24, SISP1) BTNL2 (BTL-II) oraz HHLA2 (B7-H6, B7-H7). Natomiast do receptorów białka CD28 do tej pory włączono: CD28, CTLA-4 (CD152), ICOS (CD278), PD-1 (CD279) oraz TMIGD2 (IGPR1) (Tabela 3) [15,16,18] Szlaki PD-1 oraz CTLA-4 są najlepiej poznanymi szlakami oraz aktualnie wykorzystywane w terapiach immunologicznych. Cząsteczki rodziny CD28 posiadają wspólną strukturę białkową. Składają się z pojedynczej zewnątrzkomórkowej domeny immunoglobulinowej IgV oraz cytoplazmatycznej domeny z jedną lub paroma resztami tyrozynowymi [17].

Tabela 3. Rodzina białek B7/CD28.

Ligandy (B7)	Receptory CD28
B7-1 (CD80/B7/B7.1/BB1/CD28LG/ CD28LG1/LAB7)	**CD28**
	CTLA-4 (CD152)
	PD-L1 (B7-H1/CD274/ PDCD1LG1/ B7H1/B7-H/PDCD1L1/PDCD1LG1/PDL1)
B7-2 (CD86/B7.2/B70/CD28LG2/LAB72)	**CD28**
	CTLA-4 (CD152)
B7-DC (CD273/PDCD1LG2/B7DC/ Btdc/PDCD1L2/PDL2/bA574F11.2)	**PD-1** (CD279)
	RGMb
B7-H1 (PD-L1/CD274/PDCD1LG1/B7H1/ B7-H/PDCD1L1/PDCD1LG1/PDL1)	**PD-1** (CD279)
	B7-1 (CD80/B7/B7.1/BB1/ CD28LG/CD28LG1/LAB7)
B7-H2 (CD275/ICOSLG/B7H2/ B7RP-1/B7RP1/GL50/ICOS-L/ICOSL/LICOS/)	**ICOS** (CD278)
B7-H3 (CD276/4Ig-B7-H3/B7H3/B7RP-2)	Nieznany
B7-H4 (B7x/B7s/VTCN1/B7H4/B7S1/B7X/ B7h.5/PRO1291/VCTN1)	Nieznany
B7-H5 (VISTA/VSIR/B7H5/GI24/PP2135/ SISP1/DD1alpha/C10orf54//PD-1H/)	Nieznany
B7-H6 (NCR3LG1)	**NKp30** (NCTR3/CD337)
B7-H7 (BTNL2/BTL-II/HHLA2/ B7H7/B7-H5/B7y)	**CD28H** (TMIGD2/IGPR-1)

4.1.1 Szlak PD-L1 (B7-H1)/PD-L2 (B7-DC)/PD-1

B7-H1 (PD-L1) i B7-DC (PD-L2)

Białko B7-H1 lub ligand programowanej śmierci komórki PD-L1 (ang. *programmed cell death ligand 1*) jest białkiem błonowym typu I (masa 40kDa), kodowanym przez gen *CD274* na chromosomie 9 (locus 9p24.1). PD-L1 odgrywa rolę w aktywacji i regulacji układu odpornościowego. Występuje na wielu komórkach układu odpornościowego (limfocytach T, komórkach dendrytycznych, limfocytach B, limfocytach NK, monocytach, makrofagach) i innych komórkach (komórkach śródbłonka, nabłonka, fibroblastach, mezenchymalnych komórkach macierzystych, syncytiotrofoblastach, wyspach Langerhansa, neuronach). Białko PD-L1 bierze udział w różnicowaniu limfocytów T. Poziom PD-L1 wzrasta w wielu nowotworach (czerniaku, glejaku, raku płaskonabłonkowym jajnika, głowy i szyi, sutka, esicy, trzustki i niedrobnokomórkowego raka płuca), a także w stanach związanych z przewlekłym stanem zapalnym. Niektórzy autorzy sugerują, że szlak PD-L1/PD-1 ma znaczenie prognostyczne dla przeżycia pacjentów z rakiem płaskonabłonkowym jamy ustnej [19].

Białko B7-DC lub ligand programowanej śmierci komórki PD-L2 (ang. *programmed cell death ligand 2*) jest kodowany przez gen *PDCD1LG2* na chromosomie 9 (locus 9p24.1). Transbłonowe białko PD-L2 występuje głownie w komórkach dendrytycznych, hepatocytach oraz niektórych grupach makrofagów. Jedną z funkcji cząsteczki jest hamowanie odpowiedzi komórek T poprzez wiązanie PD-1. Występowanie tego białka na komórkach nowotworowych może być związane z odpornością guza na działanie układu immunologicznego [20].

Białka PD-L1 i PD-L2 posiadają immunoglobulinowe domeny zewnątrzkomórkowe IgV i IgC, które są homologiczne tylko z PD-1 [21]. Fragmenty zewnątrzkomórkowe PD-L1 i PD-L2 mają struktury podobne do immunoglobulin, natomiast struktury wewnątrzkomórkowe wciąż są słabo poznane [22-24]. Białka PD-L1 działają supresyjnie na układ odpornościowy [25]. Badania wskazują, że cząsteczka PD-L1 odgrywa kluczową rolę w różnicowaniu regulatorowych limfocytów T. Wzrost statusu białka PD-L1 powiązany jest z przewlekłymi stanami zapalnymi i wydzielaniem interferonu γ (IFN-γ; np. w zapaleniu wątroby typu B) [26,27,28].

Ekspresję białka PD-L1 znaleziono w błonie komórkowej wielu rodzajów nowotworów, między innymi: glejaka, raka jajnika, nerki, płaskonabłonkowego głowy i szyi, sutka, esicy, niedrobnokomorkowego raka płuca oraz czerniaka [18]. Lin YM, i wsp. [29] sugerują możliwość wykorzystywania białka PD-L1 jako czynnika prognostycznego szczególnie u osób palących i mężczyzn, pomimo że statystycznie częściej PD-L1 występowało w guzach u kobiet. O cząsteczce PD-L2 i jej wpływie na immunologię nowotworów wiadomo niewiele. W raku jamy ustnej stwierdzono korelację pomiędzy wysoką ekspresją PD-L1 a dobrym całkowitym przeżyciem [30]. Natomiast w badaniach Stasikowskiej-Kanickiej O, i wsp. [31,32] wysoka ekspresja PD-L1 może świadczyć o złej prognozie.

PD-1

Receptor programowanej śmierci PD-1 (ang. *programmed death receptor 1)* o masie cząsteczkowej 50-55 kDa jest transbłonowym białkiem kodowanym przez gen *PDCD1* w chromosomie 2 (locus 2q37.3). Receptor składa się z pięciu domen, w tym z domeny zewnątrzbłonowej IgV-podobnej i wewnątrzbłonowych: ITIM (ang. *immunoreceptor tyrosine-based inhibitory motif*) oraz ITSM (ang. *immunore- ceptor tyrosine-based switch motif*). Domena IgV-podobna wykazuje podobieństwo genowe w 21-33% do CTLA-4 (antygen-4 związany z limfocytem T, ang. c*ytotoxic T-lymphocyte-associated protein 4*) [20]. Receptor PD-1 występuje w błonie komórkowej aktywowanych limfocytów T, B, NK, komórek tucznych, makrofagów i komórek dendrytycznych (ang. *dendritic cells*) [33]. Nie wykryto receptorów PD-1 w niedojrzałych limfocytach T. Stwierdzono wyższe stężenie białka PD-1 na TILs w porównaniu do krwi obwodowej, niezależnie od wieku pacjenta czy infekcji HPV [34]. Receptor PD-1 generuje sygnał hamujący, który reguluje funkcje limfocytów T. PD-1 ma dwa znane ligandy: PD-L1 (B7-H1, CD274) i PD-L2 (B7-DC, CD273), które nie ulegają wiązaniu z innymi receptorami rodziny CD28 [35]. Mimo nazwy, receptor programowanej śmierci PD-1, nie wpływa bezpośrednio na apoptozę lub przeżycie komórki. Sygnały PD-1 regulują odpowiedź komórkową, natomiast nie jest ona do końca jasna. Proces przekazywania sygnału jest różny w limfocytach B i T. Po związaniu przez receptor PD-1 ligandu następuje fosforylacja tyrozyny w domenie ITSM i rekrutacja cząsteczki sygnałowej SHP-2 (ang. *Src homology region 2 domain-*

containing phosphatase-2) i SHP-1 (ang. *Src homology region 2 domain-containing phosphatase-1*). Powoduje to blokowanie aktywacji cząsteczek PI-3K (ang. *phosphatidyl-inositol 3-kinase*) i ZAP70 (ang. *zetachain-associated protein kinase 70*). Aktywacja SHP-2 indukuje defosforylację molekuły zaangażowanej w aktywność receptora TCR (ang. *T cell receptor*), w rezultacie osłabia sygnał i redukuje syntezę cytokin [35]. Szersze rozpowszechnienie PD-1 na aktywowanych komórkach limfatycznych sugeruje, że jest to mechanizm istotniejszy niż szlak receptora CTLA-4 [36]. Reakcja zapalna wywołuje wzrost ekspresji PD-L1 i PD-L2, które mogą służyć jako mechanizm sprzężenia zwrotnego w celu zmniejszenia odpowiedzi komórek T w tkankach, chroniąc je przed uszkodzeniami z autoagresji. Połączenie PD-1 z PD-L1 prowadzi do zahamowania proliferacji i zmniejszenia sekrecji cytokin IFN-γ i IL-10 (interleukina 10) o około 80%, a IL-2 (interleukina 2) do progu poniżej wartości referencyjnych, co dodatkowo hamuje proliferację limfocytów i sprzyja przeżyciu komórki nowotworowej [37]. Przy aktywnych limfocytach poziom PD-1 jest wysoki, jednak szybko ulega zmniejszeniu po usunięciu antygenu. Gdy jednak limfocyty muszą zmagać się z przewlekłym zapaleniem (infekcja lub zmiana nowotworowa), ekspresja PD-1 jest ciągle wysoka, co powoduje „wyczerpanie" limfocytów. Wysoki poziom estrogenów może również indukować PD-1 na komórkach T i APC [38]. Efekt hamujący stwierdzono na komórkach Th (CD4+) oraz Tc (CD8+) [39]. Rola szlaku PD-1/PD-L1 nie ogranicza się do patogenezy chorób nowotworowych, ale ma znaczenie w rożnych jednostkach chorobowych: cukrzycy insulinozależnej, toczniu rumieniowatym, zapaleniu mięśnia sercowego, zapaleniu mózgu i rdzenia kręgowego, reumatoidalnym zapaleniu stawów i zapalnych chorobach jelit [40].

4.2 Interleukina 33

IL-33 (interleukin 33) jest jednym z białek rodziny IL-1 (IL-1α, IL-1β, IL-1RA, IL-18, IL-36Ra, IL-36α, IL-37, IL-36β, IL-36γ, IL-38 i IL-33). IL-33 jest kodowane przez gen *IL-33* na chromosomie 9 (locus 9: 6.22-6.26). Białko IL-33 jest zlokalizowane w jądrze i związane z chromatyną. Pro-IL-33 jest biernie uwalniana do przestrzeni zewnątrzkomórkowej, głównie przez obumierające komórki. Rodzina IL-1 odpowiada za funkcje prozapalne i przeciwzapalne. W warunkach normalnych białko IL-33 znajduje się na komórkach śródbłonka, ale także może być produkowane

wpływem różnych czynników stresogennych. Może działać jako interleukina kompleksu IL-33/ST2 (ang. *membrane receptor soluble interleukin 1 receptor-like 1,* ST2), ale także jako czynnik wewnątrzkomórkowy [41]. Białko IL-33 jest aktywowane przez kaspazy, co odróżnia ją od innych białek grupy IL-1. Działa także jako biologicznie aktywny czynnik o pełnej długości. Wiele komórek może syntetyzować IL-33 i magazynować w swoim jądrze. Wydzielanie IL-33 było głównie opisywane w procesach gojenia się ran i po urazach, gdzie IL-33 odgrywało rolę cytokiny prozapalnej. Ponadto białko IL-33 jest zaangażowane w procesy związane z przewlekłymi stanami zapalnymi, takimi jak astma, choroba Leśniowskiego-Crohna, infekcje bakteryjne, grzybicze i pasożytnicze [41,42]. Badania pokazują wpływ IL-33 i jej receptora ST2 jako modulatorów mikrośrodowiska guza (TME). IL-33/ST2 prawdopodobnie reguluje fibroblasty towarzyszące TME (ang. *carcinoma-associated fibroblasts,* CAF), które odpowiadają za progresję nowotworu (indukują migrację, przemianę nabłonkowo-mezenchymalną, inwazję i proliferację komórek) oraz aktywację limfocytów T pomocniczych. Szlak IL-33/ST2 pośrednio reguluje produkcję IL-4, IL-5 i IL-13. Wiąże się to z angiogenezą sprzyjającą przerzutom nowotworów złośliwych. Wydaje się, że nadekspresja IL-33 wykazuje zarówno działanie przeciwnowotworowe (np. w raku trzustki, jajnika, jelita grubego), jak i pro-nowotworowe (np. w raku sutka, płuca) [43-45].

4.3 Szlak PD-L1 (B7-H1)/PD-L2 (B7-DC)/PD-1 a IL-33

Mikrośrodowisko nowotworu jest dynamicznym ekosystemem składającym się z komórek nowotworowych, komórek zrębu, naczyń krwionośnych, macierzy zewnątrzkomórkowej i wielu typów komórek odpornościowych, które wykazują złożone wzajemne oddziaływania [46,47]. Komórki nowotworowe i komórki odpornościowe mogą oddziaływać poprzez białko PD-L1 i jego receptor – PD-1 [48]. Ekspresja białka PD-L1 przez komórki nowotworowe jest jednym z mechanizmów unikania odpowiedzi immunologicznej, ponieważ aktywuje ona białko PD-1 immunologicznego punktu kontrolnego na cytotoksycznych limfocytach T CD8+ i zmniejsza ich aktywność (zjawisko „wyczerpania" limfocytów) [49]. W niektórych nowotworach ekspresja PD-L1 na komórkach układu odpornościowego wiąże się z korzystnym rokowaniem, podczas gdy w innych jest złym czynnikiem prognostycznym [50,51]. Szlak białek PD-L1/PD-1 jest celem wielu leków (inhibitorów immunologicznych punktów kontrolnych), które znacząco poprawiają przeżywalność pacjentów, np. w zaawansowanym niedrobnokomórkowym raku płuca czy czerniaku [19,52,53]. Do tej pory kilka badań miało na celu ocenę prognostycznego znaczenia szlaku PD-L1/PD-1 w raku jamy ustnej [12]. Połączenie immunoterapii anty-PD-1 (pembrolizumab lub niwolumab) z chemioterapią i radioterapią poprawia wyniki leczenia w raku jamy ustnej [54].

Interleukina-33 to cytokina uwalniana z jądra do przestrzeni zewnątrzkomórkowej pod wpływem stresu lub martwicy, pełniąca funkcję endogennego sygnału zagrożenia (alarmina) [55]. IL-33 odpowiada za aktywację układu odpornościowego w odpowiedzi na uszkodzenie tkanki poprzez interakcje z jego receptorem ST2, prezentowanym przez różne komórki odpornościowe. Szlak IL-33/ST2 wpływa na elementy TME, ponieważ modyfikuje aktywność limfocytów T pomocniczych, reguluje produkcję IL-4, IL-5 i IL-13 oraz angiogenezę [56,57]. Białko IL-33 reguluje ekspresję białek PD-1/PD-L1 w mikrośrodowisku nowotworu, a wstępne badania sugerują, że wspólne ukierunkowanie na receptory IL-33 i punkty kontrole układu odpornościowego mogą poprawić wyniki immunoterapii [58].

5. CELE PRACY

Głównym celem pracy była analiza immunohistochemiczna wybranych czynników immunomodulujących w raku płaskonabłonkowym jamy ustnej.

Do celów szczegółowych należą:

1 Ocena poziomu białek PD-L1 i IL-33 na komórkach nowotworowych i na limfocytach naciekających guz w raku płaskonabłonkowym jamy ustnej.
2 Analiza zależności między cechami klinicznymi i patologicznymi a statusem poszczególnych białek w raku jamy ustnej.
3 Ocena zależności między białkami PD-L1 i IL-33 a całkowitym przeżyciem pacjentów w raku płaskonabłonkowym jamy ustnej.
4 Określenie wzajemnych zależności pomiędzy białkiem PD-L1 i IL-33.

6. MATERIAŁ I METODY

6.1 Grupa badana

Badaną grupę stanowiło 98 pacjentów (mężczyźni i kobiety w wieku 36-89 lat) z rakiem płaskonabłonkowym jamy ustnej, którzy byli leczeni w Uniwersyteckim Centrum Klinicznym Gdańskiego Uniwersytetu Medycznego w latach 2007-2012. Badanie uzyskało pozytywną opinię Niezależnej Komisji Bioetycznej Gdańskiego Uniwersytetu Medycznego (NKBBN/59/2016).

Kryteriami włączenia były: potwierdzony histopatologicznie pierwotny rak płaskonabłonkowy jamy ustnej, dostępny materiał histopatologiczny, zgoda na badanie oraz pełna dokumentacja medyczna. Kryteriami wyłączenia były: brak zgody pacjenta na badanie oraz niepełna dokumentacja medyczna. Do badania wykorzystano zarchiwizowane preparaty raka płaskonabłonkowego jamy ustnej, które zostały pobrane podczas zabiegów chirurgicznego usunięcia nowotworu lub w postaci wycinków, a jednocześnie nie były poddane działaniu radio- lub chemioterapii. Bloczki parafinowe były przechowywane i badane w Zakładzie Patomorfologii Gdańskiego Uniwersytetu Medycznego (kierownik prof. dr hab. n. med. Wojciech Biernat). W przebiegu badania zachowano pełną poufność danych.

Do oceny stopnia zaawansowania patomorfologicznego oraz klinicznego zastosowano 8 edycję klasyfikacji TNM (Tabela 1 i 2) [10]. Stopień zróżnicowania histopatologicznego analizowano w skali 4-stopniowej (G1-G4). Ponadto przeanalizowano dokumentację medyczną (wiek w momencie rozpoznania, płeć, używki, występowanie chorób przewlekłych i nowotworów w rodzinie, czas wystąpienia objawów i zgłoszenia się celem leczenia, lokalizację nowotworu, stopień zaawansowania nowotworu wg klasyfikacji TNM, stopień zaawansowania histopatologicznego zmiany, leczenie, wystąpienie wznowy oraz całkowity czas przeżycia.

6.2 Mikromacierze tkankowe

Badany materiał histopatologiczny został oceniony makroskopowo. Kolejnym etapem było przygotowanie pierwotnych bloków parafinowych poprzez utrwalenie tkanek w zbuforowanej 10% formalinie, a następnie rutynowa procedura zatopienia w parafinie. Pod kontrolą mikroskopu świetlnego wybrano reprezentatywne fragmenty zawierające tkanki raka płaskonabłonkowego jamy ustnej. Z bloków pierwotnych wybrano po 2 rdzenie tkankowe o średnicy 0,4 cm (Manual Tissue Arrayer MTA 1 Beecher Instruments Inc, Sun Prairie, USA), które umieszczono we wtórnym bloku parafinowym i uzyskano mikromacierze tkankowe (ang. *tissue microarray*, TMA). W kolejnym etapie TMA zostały pocięte na mikrotomie na skrawki średnicy 4 μm i umieszczone na szkiełkach podstawowych.

6.3 Badanie immunohistochemiczne

Barwienie immunohistochemiczne (IHC) przeprowadzono przy użyciu systemu Ventana G11 (CONFIRM™, Ventana Medical Systems, Tucson, AZ, USA).W badaniu wykorzystano monoklonalne przeciwciała królicze przeciwko PD-L1 (E1L3N® Cell Signaling, Danvers, MA, USA) XP® i IL-33 (MAB36252; Clone 1061A; R&D Systems, Inc., Bio-Techne, MN, USA). Kontrolę pozytywną stanowiły prawidłowe migdałki podniebienne.

Barwienie immunohistochemiczne składało się z:

1. Deparafinacji preparatów z wykorzystaniem ksylenów
2. Płukania w wodzie destylowanej przez 3 minuty po każdym roztworze ksylenów
3. Nawodnienia roztworami alkoholi: 100%, 96%, 80%, 70%
4. Płukania w wodzie destylowanej przez 3 minuty po każdym roztworze z szeregu alkoholi
5. Odsłonięcia epitopów (odzyskiwanie antygenowości z zastosowaniem metody odsłaniania epitopów HIER – urządzeniu typu *pressure cooker,* ciśnienie 120 hPa, bufor firmy DAKO pH 6,1, przez 1,5 godziny)
6. Chłodzenia preparatów przez 20-30 minut w temperaturze pokojowej

7. Umieszczenia próbek w komorze wilgotnej do IHC
8. Płukania preparatów przez 3 minuty roztworem buforu fosforanowego PBS (ang. *phosphate buffered saline*)
9. Neutralizacji przez 3 minuty endogennej peroksydazy za pomocą 3% nadtlenku wodoru (*Peroxidase Block RE7101*)
10. Płukania próbek roztworem PBS przez 3 minuty
11. Inkubacji preparatów przez 5 minut z 0,4% roztworem fosforanowym kazeiny (*Protein Block RE7102*)
12. Płukania preparatów roztworem PBS przez 3 minuty
13. Inkubacji próbek przez 1,5 godziny z przeciwciałami pierwotnymi w temperaturze pokojowej
14. Płukania preparatów roztworem PBS przez 3 minuty
15. Inkubacji przez 30 minut z 10% surowicą zwierzęcą (*Post Primary Block RE7111*)
16. Płukania preparatów roztworem PBS przez 3 minuty
17. Inkubacji przez 30 minut z przeciwciałami wtórnymi (*NovoLink Polimer RE7112*)
18. Płukania preparatów roztworem PBS przez 3 minuty
19. Ekspozycji przez 5 minut z 3,3'-diaminobenzydyną (DAB)
20. Płukanie preparatów roztworem PBS przez 3 minuty
21. Barwienie próbek przez 5 minut hematoksyliną Mayera
22. Dehydratacji preparatów w rosnącym szeregu alkoholowo-ksylenowym (70%, 80%, 96%, 100%)
23. Przykrycia próbek pod szkiełkiem nakrywkowym przy pomocy balsamu kanadyjskiego.

Przygotowane preparaty oceniono w mikroskopie świetlnym. Proporcję komórek z pozytywną ekspresją ustalono przez obliczenie liczby wybarwionych komórek nowotworowych (TCs) i limfocytów naciekających nowotwór (TILs) podzielonych przez całkowitą liczbę komórek każdego typu. Dla każdego pacjenta wykorzystano wyniki z dwóch rdzeni. W każdym rdzeniu oszacowano odsetek dodatnio barwiących się komórek i w dalszych analizach wykorzystano średni wynik. Punkt

odcięcia dla wysokiej ekspresji PD-L1 ustalono w zależności od mediany odsetka dodatnio barwiących się komórek jako > 10% w TCs i > 20% w TILs. Ekspresję białka IL-33 podzielono na dwie grupy — brak ekspresji i pozytywna ekspresja. Następnie porównano zgodność między dwoma rdzeniami w klasyfikacji binarnej PD-L1 i IL-33 według współczynnika kappa Cohena w celu oceny heterogeniczności ekspresji markerów. Ekspresję białka PD-L1 i IL-33 oceniono u 95 pacjentów (trzech pacjentów wyłączono z badania z powodu utraty rdzenia TMA).

6.4 Analiza statystyczna

Analizę statystyczną wykonano przy użyciu programu STATISTICA 13.3 (TIBCO, Palo Alto, CA, USA) i *R statistical enviroment*. Związki między analizowanymi markerami a cechami kliniczno-patologicznymi oceniano testem U Mann–Whitney dla zmiennych ciągłych. Zmienne kategorialne porównywano testem chi-kwadrat i testem Fishera. Współczynnik kappa Cohena obliczono, aby ocenić powtarzalność barwienia na dwóch rdzeniach TMA. Analizę całkowitego przeżycia całkowitego (ang. *overall survival,* OS) analizowano metodą Kaplana-Meiera i porównano za pomocą testu log-rank lub testu F-Cox. Przyjęto poziom istotności statystycznej $p \leq 0{,}05$.

7. WYNIKI

7.1 Charakterystyka badanej grupy

Większość pacjentów stanowili mężczyźni (67%). Średni wiek badanych wynosił około 62 lata (zakres od 36 do 89 lat). Najliczniejszą grupę stanowili chorzy z nowotworem w stadium T2 (23,47%) i T1 (18%). Brak przerzutów do lokalnych węzłów chłonnych stwierdzono u 42% pacjentów. U żadnego z pacjentów nie wykryto przerzutów odległych w momencie rozpoznania. Trzydzieści dwa procent chorych prezentowała IV stopnień zaawansowania klinicznego nowotworu. Nikotynizm i alkoholizm deklarowało odpowiednio 52% i 14% badanych. Leczenie chirurgiczne było najczęściej stosowaną metodą terapii. Szczegółowe dane kliniczno-patologiczne przedstawia Tabela 4.

Ponad 62% pacjentów nie przeżyła 5 lat. W badaniu przeanalizowano czas całkowitego przeżycia przy użyciu modelu proporcjonalnego ryzyka regresji wg Cox'a. W analizie jednoczynnikowej czas przeżycia był powiązany z wiekiem (p = 0,040), stopniem zaawansowania klinicznego (p = 0,001), wielkością guza pierwotnego (p = 0,023), obecnością przerzutów do lokalnych węzłów chłonnych (p = 0,010), leczeniem chirurgicznym (p = 0,003) i czasem od wystąpienia objawów do diagnozy (p = 0,002; Tabela 5). W przypadku pacjentów w podeszłym wieku, w zaawansowanym stadium T i N, u których nie przeprowadzono radykalnego leczenia chirurgicznego i z długim czasem od wystąpienia objawów do diagnozy, obserwowano krótszy czas całkowitego przeżycia. W analizie wieloczynnikowej całkowity czas przeżycia był związany z leczeniem operacyjnym (p = 0,0064) i czasem od wystąpienia objawów do diagnozy (p = 0,001). Pięcioletnie przeżycie wolne od choroby było związane ze stopniem zaawansowania (p = 0,003), stopniem T (p = 0,035), stopniem N (p = 0,047) i leczeniem chirurgicznym guza pierwotnego (p = 0,017). Stopień T1 i T2, brak przerzutów do lokalnych węzłów chłonnych N0 oraz radykalne leczenie operacyjne wiązały się z pięcioletnim DFS. W wieloczynnikowej analizie regresji Cox'a leczenie chirurgiczne dwukrotnie zwiększyło całkowity czas przeżycia. Dłuższy czas od wystąpienia objawów do diagnozy (> 7 miesięcy) zmniejszał przeżywalność 1,5 raza.

Tabela 4. Dane kliniczno-patologiczne (* Test Mann-Whitney; ** Test Kruskala-Wallisa).

Dane kliniczno-patologiczne	Wszyscy pacjenci n (%) 98 (100)
Wiek*	
<60	39 (39,80)
≥60	59 (60,20)
Płeć*	
Mężczyźni	66 (67,35)
Kobiety	32 (32,65)
Palenie tytoniu**	
Osoby palące	51 (52,04)
Osoby niepalące	22 (22,45)
Brak danych	25 (25,51)
Nadużywanie alkoholu*	
Osoby nadużywające alkohol	14 (14,29)
Osoby nienadużywające alkoholu	58 (59,18)
Brak danych	26 (26,53)
Nowotwór w rodzinie*	
Tak	4 (4,08)
Nie	67 (68,37)
Brak danych	27 (27,55)
Czas od objawów do rozpoznania**	
1-3 miesiące	23 (23,47)
3-5 miesięcy	4 (4,08)
5-7 miesięcy	8 (8,16)
> 7 miesięcy	8 (8,16)
Brak danych	55 (56,12)
Stopień zróżnicowania histopatologicznego**	
G1	38 (38,78)
G2	49 (50,00)
G3	11 (11,22)
System TNM	
T**	
T1	18 (18,37)
T2a, T2b	23 (23,47)

Dane kliniczno-patologiczne	Wszyscy pacjenci n (%) 98 (100)
T3	12 (12,24)
T4a, T4b	15 (15,31)
Brak danych	30 (30,61)
N*	
N0	41 (41,84)
N1	10 (10,20)
N2a, N2b, N2c	13 (13,27)
N3a, T3b	4 (4,08)
Brak danych	30 (30,61)
M*	
M0	4 (4,08)
M1	0 (0,00)
Mx	63 (64,29)
Brak danych	31 (31,63)
Stopień zaawansowania wg WHO**	
I	18 (18,37)
II	15 (15,31)
III	13 (13,27)
IV	31 (31,63)
Brak danych	21 (21,43)
Lokalizacja**	
Język i dno jamy ustnej	55 (56,12)
Pozostałe lokalizacje (dziąsło górne, dziąsło dolne, podniebienie twarde, trójkąt zatrzonowcowy)	43 (43,88)
Leczenie chirurgiczne*	
Tak	62 (63,27)
Nie	20 (20,41)
Brak danych	16 (16,33)
Radioterapia*	
Tak	41 (41,84)
Nie	36 (36,73)
Brak danych	23 (21,43)
Chemioterapia*	
Tak	9 (9,18)

Dane kliniczno-patologiczne	Wszyscy pacjenci n (%) 98 (100)
Nie	68 (69,39)
Brak danych	21 (21,43)
Wznowa*	
Tak	15 (15,31)
Nie	57 (58,16)
Brak danych	26 (26,53)

Tabela 5. Analiza przeżycia całkowitego i pięcioletniego przeżycia wolnego od choroby (HR – współczynnik ryzyka; 95% CI – przedział ufności; *p* – wartość *p*).

Całkowite przeżycie				**Pięcioletnie przeżycie bez choroby**	
Jednoczynnikowa analiza regresji Cox'a	**HR**	**95% CI**	***p***	**Statystyka**	***p***
Dane kliniczno-patologiczne					
Wiek	1,024	1,001-1,048	**0,040**	Chi-kwadrat	0,527
Płeć	0,974	0,599-1,584	0,916	Chi-kwadrat	1,000
Palenie tytoniu	1,378	0,734-2,587	0,319	Chi-kwadrat	0,550
Alkoholizm	0,920	0,447-1,893	0,820	Chi-kwadrat	1,000
Nowotwór w rodzinie	2,746	0,961-7,845	0,059	Fisher	0,126
Czas od objawów do diagnozy	1,372	1,12-1,681	**0,002**	Fisher	0,206
Stopień zróżnicowania histopatologicznego	1,325	0,948-1,851	0,100	Fisher	0,785
T	1,345	1,042-1,737	**0,023**	Chi-kwadrat	**0,035**
N	1,428	1,088-1,874	**0,010**	Fisher	**0,047**
M	0,729	0,405-1,312	0,292	Fisher	0,618
Stopień zaawansowania wg WHO	1,452	1,158-1,82	**0,001**	Chi-kwadrat	**0,003**
Lokalizacja	1,000	1,000-1,000	**0,583**	-	**-**
Leczenie chirurgiczne	0,430	0,245-0,754	**0,003**	Chi-kwadrat	**0,017**
Radioterapia	1,524	0,887-2,618	0,127	Chi-kwadrat	0,298
Chemioterapia	0,825	0,353-1,926	0,656	Fisher	1,000
Wznowa	0,690	0,345-1,381	0,295	Chi-kwadrat	0,056
Wieloczynnikowa analiza regresji Cox'a					
Dane kliniczno-patologiczne					
Czas od objawów do diagnozy	1,450	1,15-1,744	**0,001**		
T	1,240	0,47-3,22	0,665		
N	1,360	0,81-2,28	0,239		
Stadium zaawansowania wg WHO	0,860	0,86-0,38	0,714		
Leczenie chirurgiczne	0,400	0,209-0,774	**0,0064**		

7.2 Immunohistochemiczna ocena białka PD-L1

W czterdziestu czterech (46,31%) przypadkach wykazano pozytywną ekspresję białka PD-L1 w TCs (wysoka ekspresja > 10%). Średni odsetek komórek dodatnich wynosił 21,88%, mediana 10%. Guzy zlokalizowane na dnie jamy ustnej i języku miały zwykle mniejszy odsetek PD-L1-dodatnich TCs w porównaniu z innymi lokalizacjami (p = 0,019, U Mann–Whitney). Zaobserwowano zależność pomiędzy niższym poziomem ekspresji białka PD-L1 oraz obecnością przerzutów do lokalnych węzłów chłonnych (p = 0,015, U Mann–Whitney). Natomiast nie wykazano związku pomiędzy ekspresją białka a płcią, stadium T, stopniem zaawansowania, paleniem papierosów lub nadużywaniem alkoholu (Tabela 6).

Trzydzieści jeden (31,63%) przypadków wykazało dodatni wynik PD-L1 w TILs (wysoka ekspresja > 20%). Wysoka ekspresja PD-L1 na TILs była związana z brakiem przerzutów do węzłów chłonnych i niższym stadium zaawansowania klinicznego. Nie wykazano zależności pomiędzy ekspresją białka PD-L1 w TILs a pozostałymi danymi kliniczno-patologicznymi.

Zgodność między rdzeniami była umiarkowana pod względem ekspresji białka PD-L1 w TCs (kappa Cohen = 0,645, 95% CI = 0,492–0,799) i zadowalająca pod względem ekspresji w TILs (kappa Cohen = 0,335, 95% CI = 0,126–0,545).

Tabela 6. Zestawienie cech kliniczno-patologicznych w odniesieniu do ekspresji PD-L1 na TCs i TILs (*p* – *p* wartość; * — istotna statystycznie *p*). Wartości *p* obliczono za pomocą chi-kwadrat.

Dane kliniczno-patologiczne	Pacjenci n (%)	PD-L1 na TCs			PD-L1 na TILs		
		Niska ekspresja n (%)	Wysoka ekspresja n (%)	*p*	Niska ekspresja n (%)	Wysoka ekspresja n (%)	*p*
Płeć							
Kobiety	32 (33,68)	16 (16,84)	16 (16,84)	0,607	20 (21,05)	12 (12,63)	0,292
Mężczyźni	63 (66,32)	35 (36,84)	28 (29,47)		46 (48,42)	17 (17,89)	
Palenie tytoniu							
Nie	22 (30,14)	11 (15,07)	11 (15,07)	0,939	15 (20,55)	7 (10,27)	0,548
Tak	51 (69,86)	26 (35,62)	25 (34,25)		31 (42,47)	20 (27,40)	
Alkoholizm							
Nie	58 (80,56)	29 (40,28)	29 (40,28)	1,000	35 (48,61)	23 (31,94)	0,415
Tak	14 (19,44)	7 (9,72)	7 (9,72)		10 (13,70)	4 (5,56)	
Stopień zróżnicowania histopatologicznego							
1	39 (42,05)	22 (23,16)	17 (17,89)	0,656	26 (27,37)	13 (13,68)	0,620
2–3	56 (58,95)	29 (30,53)	27 (28,42)		40 (42,11)	16 (16,84)	
Stopień zaawansowania wg WHO							
I–II	32 (42,67)	17 (22,08)	15 (15,79)	0,832	14 (18,18)	18 (18,18)	0,004 *
III–IV	45 (57,33)	25 (32,47)	20 (25,97)		34 (44,16)	11 (14,29)	
T							
1–2	42 (55,26)	23 (30,26)	18 (23,68)	0,874	23 (30,26)	19 (25,00)	0,157
3–4	34 (44,74)	18 (23,68)	16 (21,05)		24 (31,58)	10 (10,53)	
N							
0	39 (52,70)	17 (22,97)	22 (29,73)	0,097	18 (24,32)	21 (28,38)	0,006*
1–3	35 (47,30)	22 (29,73)	13 (17,57)		27 (36,49)	8 (10,81)	
Lokalizacja							
Język i dno jamy ustnej	55 (57,89)	35 (36,84)	20 (21,05)	0,022*	37 (38,95)	18 (18,95)	0,584
Pozostałe lokalizacje	40 (42,11)	16 (16,84)	24 (25,26)		29 (30,53)	11 (11,58)	

7.3 Immunohistochemiczna ocena białka IL-33

Piętnaście przypadków (15,79%) wykazało pozytywną ekspresję IL-33 w jądrach TCs (średnia 1,08%, mediana 0%, maks. 30%). Pozytywna ekspresja białka IL-33 była rzadziej notowana w obrębie guzów języka i dna jamy ustnej (p = 0,001, chi-kwadrat). W przypadkach stadiów zaawansowania 3 i 4 wykazano częstszą ekspresję białka IL-33 niż w przypadku nowotworów w niższych stopniach zaawansowania, ale wartości były na granicy istotności statystycznej (p = 0,057, chi-kwadrat). Żadne inne zmienne kliniczno-patologiczne nie wykazały zależności z białkiem IL-33 na TCs.

Dodatnią ekspresję IL-33 w TILs zaobserwowano w 18 przypadkach (18,94%). Średni odsetek wynosił 0,5% (mediana 0%, maksimum 7%). Ekspresja IL-33 w TILs była mniej powszechna w nowotworach języka i dna jamy ustnej (p = 0,055, chi-kwadrat), ale nie stwierdzono innego związku między IL-33 a zmiennymi kliniczno-patologicznymi (Tabela 7).

Powtarzalność ekspresji między rdzeniami IL-33 była istotna w przypadku TCs (kappa Cohena = 0,707, 95% CI = 0,487–0,927) i umiarkowana w przypadku TILs (kappa Cohena = 0,493, 95% CI 0,235–0,752).

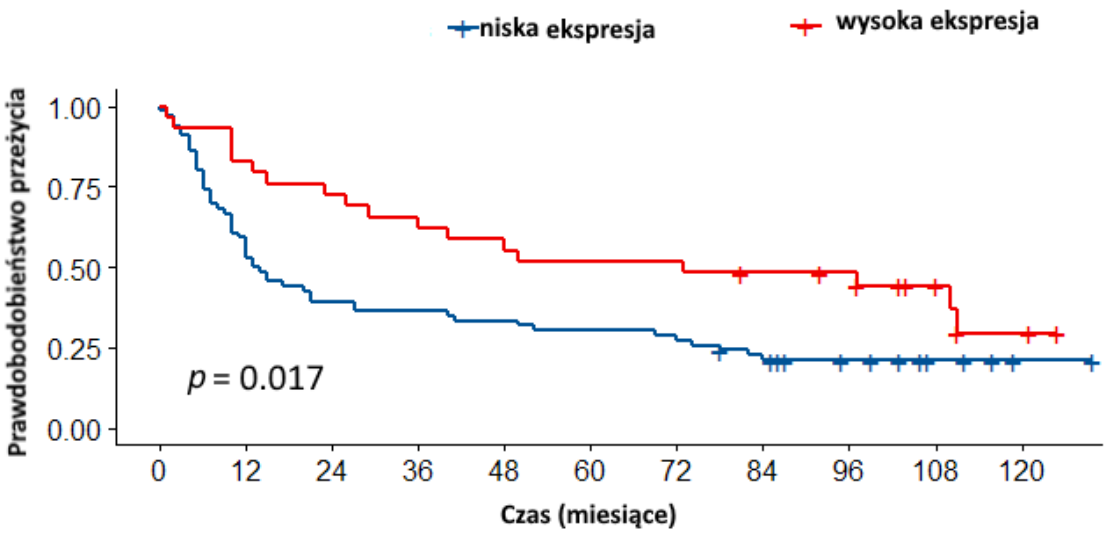
Tabela 7. Zestawienie cech kliniczno-patologicznych w odniesieniu do ekspresji IL-33 na TCs i TILs (*p* – *p* wartość*;* * — istotna statystycznie *p*). Wartości *p* obliczono za pomocą chi-kwadrat.

Dane kliniczno-patologiczne	Pacjenci n (%)	IL-33 na TCs			IL33 na TILs		
		Negatywna ekspresja n (%)	Pozytywna ekspresja n (%)	*p*	Negatywna ekspresja n (%)	Pozytywna ekspresja n (%)	*p*
Płeć							
Kobiety	32 (33,68)	29 (30,53)	3 (3,16)	0,222	28 (29,47)	4 (4,21)	0,253
Mężczyźni	63 (66,32)	51 (53,68)	12 (12,63)		48 (50,52)	14 (14,74)	
Palenie tytoniu							
Nie	22 (30,14)	16 (21,92)	6 (8,22)	0,165	16 (21,92)	6 (8,22)	0,467
Tak	51 (69,86)	44 (60,27)	7 (9,59)		41 (56,16)	10 (13,70)	
Alkoholizm							
Nie	58 (80,56)	49 (68,06)	9 (12,05)	0,594	47 (65,28)	11 (15,28)	0,427
Tak	14 (19,44)	9 (12,05)	3 (4,17)		10 (13,89)	4 (5,56)	
Stopień zróżnicowania histopatologicznego							
1	39 (42,05)	35 (36,84)	4 (4,21)	0,217	34 (35,79)	5 (5,26)	0,203
2-3	56 (58,95)	45 (47,37)	11 (11,58)		43 (45,26)	13 (13,68)	
Stopień zaawansowania wg WHO							
I-II	32 (42,67)	30 (38,96)	2 (2,60)	0,057	27 (35,06)	5 (6,49)	0,803
III-IV	45 (57,33)	35 (45,45)	10 (12,99)		37 (48,05)	8 (10,39)	
T							
1-2	42 (55,26)	37 (48,68)	5 (6,58)	0,302	34 (44,74)	8 (10,53)	0,617
3-4	34 (44,74)	27 (35,53)	7 (9,21)		29 (38,16)	5 (6,58)	
N							
0	39 (52,70)	39 (52,70)	4 (5,41)	0,142	32 (43,24)	7 (9,46)	0,927
1-3	35 (47,30)	35 (47,30)	8 (10,81)		29 (39,19)	6 (8,11)	
Lokalizacja							
Język i dno jamy ustnej	55 (57,89)	52 (54,74)	3 (3,16)	0,001*	46 (48,42)	9 (9,47)	0,451
Pozostałe lokalizacje	40 (42,11)	28 (29,47)	12 (12,63)		31 (32,63)	9 (9,47)	

7.4 Analiza przeżycia

7.4.1 Badanie całej kohorty

Jednoczynnikowa analiza proporcjonalnego hazardu Cox'a (Tabela 7) wykazała związek między przeżyciem a stopniem zróżnicowania histopatologicznego, stopniem zaawansowania, przerzutami do lokalnych węzłów chłonnych a ekspresją biała PD-L1 na TILs (Tabela 8). Ekspresja IL-33 i PD-L1 w TCs nie miała wpływu na przeżycie chorych. Wysoka ekspresja PD-L1 w TILs wiązała się z lepszym OS (HR = 0,475, 95% CI = 0,281–0,805; Rycina 1), ale nie została utrzymana w wieloczynnikowym modelu regresji Cox'a.



Rycina 1. Krzywa Kaplana-Meiera prawdopodobieństwa całkowitego przeżycia w zależności od poziomu białka PD-L1 na TILs (lepsze całkowite przeżycie).

Tabela 8. Jednowymiarowa i wielowymiarowa analiza proporcjonalnego hazardu Cox'a (HR — współczynnik ryzyka; 95% CI — przedział ufności; *p* — *p* wartość; * — istotna statystycznie *p*).

Dane kliniczno-patologiczne	**Jednoczynnikowy model proporcjonalnego ryzyka Cox'a**		**Wieloczynnikowy model proporcjonalnego ryzyka Cox'a**	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Płeć (Mężczyźni *vs*. kobiety)	1,026 (0,626–1,681)	0,919		
Palenie tytoniu (Nie *vs.* Tak)	0,738 (0,392–1,389)	0,347		
Alcohol (Nie *vs.* Tak)	1,001 (0,485–2,065)	0,997		
Lokalizacja (Język i dno jamy ustnej *vs.* pozostałe lokalizacje)	0,957 (0,596–1,537)	0,855		
Stopień zróżnicowania histopatologicznego (2–3 *vs.* 1)	1,767 (1,074–2,906)	0,025*		
Stopień zaawansowania klinicznego (1–2 *vs.* 3–4)	0,333 (0,185–0,598)	< 0,001*		
T (1–2 *vs.* 3–4)	0,383 (0,221–0,664)	< 0,001*		
N (0 *vs.* 1–3)	0,343 (0,196–0,600)	< 0,001*	0,345 (0,193–0,617)	< 0,001
PD-L1 TCs (wysoka *vs.* niska ekspresja)	0,991 (0,618–1,589)	0,971		
PD-L1 TILs (wysoka *vs.* niska ekspresja)	0,525 (0,306–0,902)	0,012*		
IL-33 TCs (pozytywna *vs.* negatywna ekspresja)	0,837 (0,428–1,637)	0,603		
IL-33 TILs (pozytywna *vs.* negatywna ekspresja)	1,036 (0,566–1,894)	0,909		

7.4.2 Znaczenie prognostyczne ekspresji PD-L1 i IL-33 w różnych lokalizacjach

Ze względu na istotne różnice w ekspresji białka PD-L1 w nowotworach zlokalizowanych w obrębie języka i dna jamy ustnej postanowiono stworzyć oddzielne wieloczynnikowe modele regresji Cox'a. W grupie raków języka i dna jamy ustnej do ostatecznego modelu włączono dwie zmienne: wielkość zmiany pierwotnej oraz ekspresję białka PD-L1 na TCs (Tabela 9).

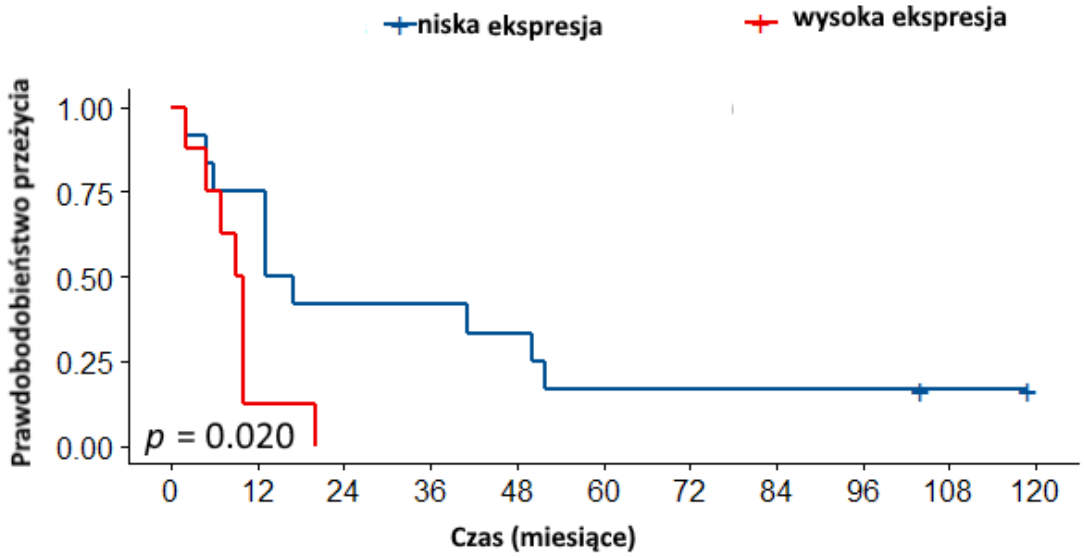
Tabela 9. Wielowymiarowe modele regresji Cox'a (HR — współczynnik ryzyka; 95% CI — przedział ufności; *p* – *p* wartość; * — istotna statystycznie *p*).

Wielowymiarowy model regresji Cox'a		
Cecha	**HR (95% CI)**	***p***
Rak jamy ustnej – język i dno jamy ustnej		
T (1–2 *vs.* 3–4)	0,229 (0,101–0,518)	< 0,001*
PD-L1 na TCs (wysoka *vs.* niska ekspresja)	2,514 (1,977–5,282)	0,014*
Inne lokalizacje		
N (0 *vs.* 1–3)	0,239 (0,081–0,699)	0,008*

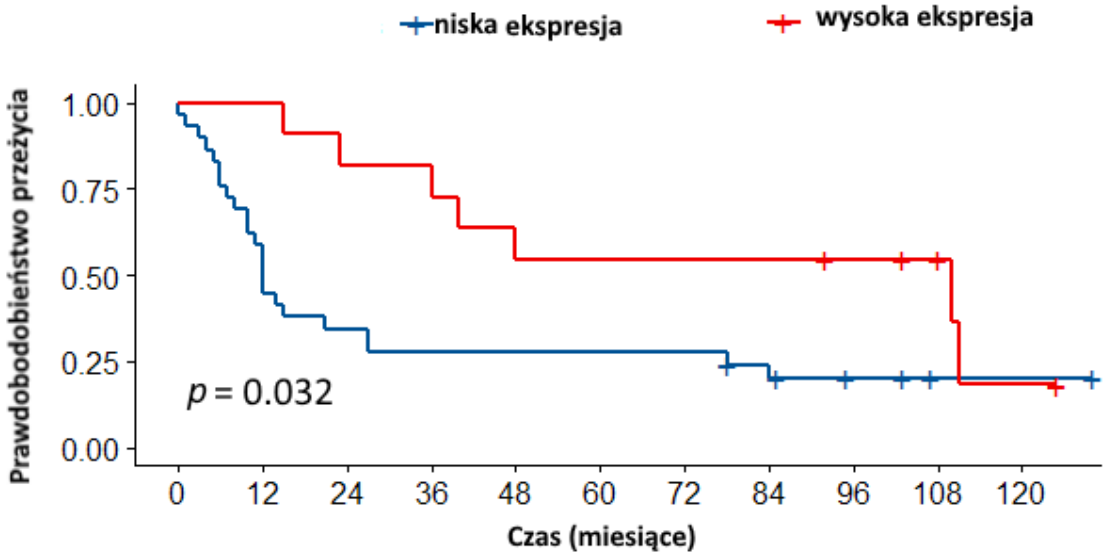
Szczególnie złe wyniki OS zaobserwowano w grupie guzów T3–4 z wysoką ekspresją PD-L1 na TCs (Rycina 2). W przypadku nowotworów zlokalizowanych w innych częściach jamy ustnej do modelu wielowymiarowego włączono jedynie obecność przerzutów do lokalnych węzłów chłonnych. W analizie jednowymiarowej ekspresja białka PD-L1 na TILs była powiązana z lepszymi wynikami przeżycia (Rycina 3).

7.5 Zależność poziomu białek PD-L1 i IL-33

Nie stwierdzono korelacji między ekspresją PD-L1 i IL-33.



Rycina 2. Krzywa Kaplana-Meiera prawdopodobieństwa przeżycia całkowitego w zależności od poziomu ekspresji białka PD-L1 na TCs w guzach T3–T4 języka i dna jamy ustnej. Nowotwory z wysoką ekspresją PD-L1 na TCs miały niski czas całkowitego przeżycia.



Rycina 3. Krzywa Kaplana-Meiera prawdopodobieństwa całkowitego przeżycia w zależności od poziomu ekspresji białka PD-L1 na TILs w pozostałych lokalizacjach jamy ustnej. Nowotwory bogate w PD-L1-dodatnie TILs miały lepsze wyniki całkowitego przeżycia chorych.

8. DYSKUSJA

Jama ustna jest w stałym kontakcie ze środowiskiem zewnętrznym. Zachodzi tu wiele reakcji, które mają na celu ochronę organizmu przed szkodliwymi czynnikami. Coraz bardziej dostrzega się znaczenie mikrośrodowiska i immunologii guza w rokowaniu pacjentów z rakiem płaskonabłonkowym jamy ustnej i innymi nowotworami złośliwymi głowy i szyi [12,13]. Jest to pierwsze badanie, w którym przeprowadzono wspólną analizę ekspresji białek PD-L1 i IL-33 na TCs i TILs w OSCC. Głównym źródłem białka IL-33 w mikrośrodowisku nowotworu są fibroblasty, komórki śródbłonka, nabłonka i guza. IL-33 aktywuje komórki tuczne, a przez to szlak chymazy/tryptazy, które uwolnione z komórek tucznych rozszczepiają pełnej długości IL-33 do wysoce aktywnej dojrzałej IL-33. Interleukina 33 (poprzez wiązanie receptora ST2 z komórkami układu odpornościowego) i aktywowane komórki tuczne (poprzez uwalnianie mediatora) wykazuje działanie na wrodzone komórki odpornościowe (eozynofile, bazofile, neutrofile, komórki supresorowe pochodzenia szpikowego, makrofagi, komórki NK, wrodzone komórki limfoidalne typu 2, komórki dendrytyczne i adaptacyjne komórki odpornościowe: limfocyty NK, limfocyty T regulatorowe, CD4, CD8 i limfocyty B). Wykazano, że białko IL-33 reguluje ekspresję PD-1/PD-L1. Aktywacja komórek tucznych przez IL-33 prowadzi do uwolnienia wielu czynników, które działają na różne typy komórek odpornościowych w środowisku guza. Poprzez PD-L1 czy TNF alfa komórki tuczne powodują rekrutację, proliferację, aktywację, różnicowanie i polaryzację limfocytów T CD8 [44,45]. IL-33 wpływa na zwiększenie ekspresji białek PD-1 i PD-L1 na powierzchni komórek T CD8 i komórek nowotworu. IL-33 zwiększa również proliferację i aktywację komórek T CD8 [57]. IL-33 zwiększa ekspresję PD-1 i PD-L1 we wrodzonych komórkach limfoidalnych 2 (ang. *innate lymphoid cells 2,* ILC2) i uczestniczy w hamowaniu niekontrolowanej proliferacji lub aktywacji ILC2 [58].

W niniejszej pracy wykazano, że poziom ekspresji PD-L1 na TCs różni się w zależności od lokalizacji – nowotwory języka i dna jamy ustnej wykazują mniejszą ekspresję białka niż nowotwory w innych lokalizacjach. Ekspresja białka PD-L1 na TCs nie była związana z całkowitym przeżyciem pacjentów, badanym w całej kohorcie. Większa ekspresja PD-L1 na TCs w nowotworach języka i dna jamy ustnej wiązała się z gorszym OS, zwłaszcza w nowotworach wyższej kategorii T wg klasyfikacji TNM. W

innych lokalizacjach wyższa ekspresja PD-L1 na TCs wiązała się z lepszym rokowaniem (zauważono tendencję, ale była nieistotna statystycznie). Z drugiej strony, wyższa ekspresja białka PD-L1 na TILs była związana z mniejszą częstością przerzutów do węzłów chłonnych. W analizie jednoczynnikowej wiązało się to dłuższym OS, ale efekt ten nie został utrzymany w analizie wieloczynnikowej. Najprawdopodobniej wynika to z bardzo silnej korelacji pomiędzy ekspresją białka PD-L1 z TILs oraz przerzutami do szyjnych węzłów chłonnych.

W innych pracach dotyczących OSCC ekspresję PD-L1 na TCs obserwowano ze zmienną częstością wahającą się od około 10% do 90% przypadków [25,26,29,30,31,32,59-87]. Badania różniły się wielkością badanej grupy, narodowością/grupą etniczną, zastosowanym klonem przeciwciał oraz sposobem oceny ekspresji białka. Może to powodować różnice w wynikach badań. Ze wszystkich przeciwciał białka PD-L1 tylko klony E1L3N, E1J2J, SP142, 28-8, 22C3 i SP263 przeszły walidację immunohistochemiczną i Western Blot. W niniejszym badaniu wykorzystano klon E1L3N. Autorzy zastosowali różne metody oceny barwienia, m.in. ocenę H-score, procent immunobarwiących się komórek lub ocenę półilościową. Różne są również poziomy odcięcia i kwalifikacja wyników jako pozytywnych. Dlatego bardzo trudne jest porównywanie badań. Ponadto większość analiz opiera się na niejednorodnej grupie guzów zlokalizowanych w całej jamie ustnej. Homogenne grupy guzów pod względem lokalizacji stanowiły badania Meehana i wsp. [61] oraz Wilmsa i wsp. [63], w których badane tkanki pochodziły z raka płaskonabłonkowego języka. Rak płaskonabłonkowy jamy ustnej może wykazywać odmienną immunobiologię ze względu na swoją lokalizację. Taki związek wykazano w tym badaniu. Chen XJ, i wsp. [87] zauważyli zależność poziomu białka PD-L1 ze wznową miejscową. W publikacjach Wilms i wsp. [63] oraz Ahmadi, i wsp. [65] wykazano, że częściej występowała ujemna ekspresja białka PD-L1 u mężczyzn niż u kobiet. W innym badaniu ekspresja białek na komórkach nowotworu była skorelowana ze stopniem zróżnicowania histopatologicznego (stopień 3 wykazywał intensywną immunoreaktywność białka PD-L1 w porównaniu z stopniem 2 i 1) [87], wielkością zmiany pierwotnej (immunoreaktywność białka PD-L1 była związana z rozmiarem guza) [67,87], przerzutami do węzłów chłonnych [87], przerzutami odległymi [87] oraz stopniem zaawansowania klinicznego (wyższy stopień związany był z wyższym

wybarwieniem) [65,66,87]. W badaniu Stasikowskiej-Kanickiej i wsp. [32] oceniano ekspresję białka PD-L1 na komórkach nowotworowych, TILs, a także komórkach śródbłonka i nabłonka. W publikacji Foy i wsp. [75] ekspresja PD-L1 była badana na komórkach guza oraz w obrębie komórek T CD8 + naciekających guz. Hirai i wsp. [76] analizowali immunohistochemicznie makrofagi i komórki dendrytyczne, które wykazywały zwiększoną ekspresję białka PD-L1 w komórkach zrębu w inwazyjnym raku jamy ustnej o wysokim stopniu złośliwości. W badaniu Hanna i wsp. [70] ekspresja białka PD-L1 na TILs była związana ze zmniejszonym ryzykiem nawrotu choroby. Natomiast w publikacji Wilmsa i wsp. [63] wykazano, że nie ma znaczących powiązań między poziomem białka PD-L1 w komórkach odpornościowych a badanymi cechami kliniczno-patologicznymi. Natomiast Ahn i wsp. [30] stwierdzili związek między ekspresją PD-L1 na komórkach nowotworowych i TILs.

W wielu badaniach oceniających przeżycie w raku płaskonabłonkowym jamy ustnej wykazano, że wysoka ekspresja PD-L1 na TCs wiąże się z gorszymi wynikami przeżycia chorych. Strati i wsp. [88] wykazali, że nadekspresja PD-L1 na krążących TCs była związana z gorszym przeżyciem bez progresji i OS w rakach głowy i szyi. Natomiast w innych badaniach przedstawiono odmienne wyniki i brak związku między ekspresją PD-L1 a przeżyciem. W niniejszym badaniu ekspresja PD-L1 na TCs wydaje się mieć różne efekty prognostyczne w różnych lokalizacjach jamy ustnej. Zależne od miejsca różnice w składzie TME raków głowy i szyi zostały wcześniej opisane przez zespół Green i wsp. [89], którzy zaobserwowali większą częstość występowania TILs w rakach części ustnej gardła w porównaniu z innymi lokalizacjami. TILs mogą odgrywać rolę w rozwoju raka jamy ustnej. Białko PD-L1 ulega fizjologicznej ekspresji na rogowaciejącej błonie śluzowej jamy ustnej, podczas gdy inne nabłonki nie wykazują konstytutywnej ekspresji PD-L1 [90]. Przedziały jamy ustnej stanowią nisze ekologiczne zamieszkałe przez różne mikroflory modulujące lokalne TME [91,92]. Na przykład *Porphyromonas gingivalis* indukuje ekspresję PD-L1 w komórkach raka płaskonabłonkowego jamy ustnej *in vitro* [26]. Niniejsze czynniki mogą przyczyniać się do odmiennych cech immunologicznych raka płaskonabłonkowego jamy ustnej w różnych lokalizacjach [93].

W badaniach dotyczących białka PD-L1 rzadko oceniano ekspresję na TILs i wpływ mikrośrodowiska guza na rozwój nowotworu. Limfocyty Tc i Th1 są

zaangażowane w skuteczną przeciwnowotworową odpowiedź immunologiczną. Lequerica-Fernández i wsp. [74] wykazali, że wysoki status TILs był związany z pozytywną ekspresją białka PD-L1 na TCs. Stosunek CD8+/FOXP3+ TILs był wyższy w TCs PD-L1-dodatnich. Wysoka infiltracja TILs CD4+ i CD8+ oraz wysoki stosunek nowotworowych CD8+/FOXP3+ były istotnie związane z obecnością pozytywnej ekspresji PD-L1 w guzie. Ponadto w innych badaniach poziom białka PD-L1 na komórkach nowotworowych był związany z całkowitym czasem przeżycia [62,70,74,77,83], przeżyciem wolnym od choroby [62,65,84], przeżyciem wolnym od progresji [70], złym rokowaniem [71] i przeżyciem wolnym od nawrotów [83]. Takahashi i wsp. [67] wykazali, że pacjenci z wysoką ekspresją PD-L1 i licznymi limfocytami T CD4+ mają lepsze wyniki przeżycia niż pacjenci z małym naciekiem limfocytów T CD4+.

W przypadku niniejszej pracy ekspresja białka IL-33 na komórkach guza i limfocytów naciekających nowotwór nie występowała często. Jak dotąd opublikowano tylko jedno doniesienie badające relacje pomiędzy białkiem IL-33 a rakiem płaskonabłonkowym jamy ustnej. W badaniu Ishikawa i wsp. [94] wykazano wysoką ekspresję interleukiny 33 na komórkach nowotworowych, którą obserwowano 50,62% pacjentów. Opisano, że ekspresja białka IL-33 na komórkach guza była związana z częstszymi przerzutami do lokalnych węzłów chłonnych oraz wznową miejscową. Ponadto udowodniono, że gorsze rokowanie występuje w przypadku grupy pacjentów z wysokim poziomem IL-33. W porównaniu niniejszej pracy do badania Ishikawy i wsp. [94] grupa badana była mniej liczna, białko IL-33 nie było analizowane na TILs oraz wykorzystano inną metodę oceny barwienia. W publikacji Ishikawa i wsp. [94] barwienie klasyfikowano poprzez oszacowanie odsetka komórek nowotworowych wykazujących specyficzną immunoreaktywność. Oceniano dwa obszary o dużej gęstości wybarwionych komórek, a następnie zliczono liczbę komórek immunoreaktywnych i całkowitą liczbę komórek nowotworowych w obu obszarach [94]. W niniejszym badaniu nie znaleziono żadnego związku między ekspresją PD-L1 i IL-33 w badanej grupie chorych.

Białko IL-33 badano także w innych nowotworach, np. w przypadku raka głowy i szyi oraz guzów ślinianek [95-97]. Wen i wsp. [95] zaobserwowali, że poziom białka

IL-33 w zrębie ma wartość prognostyczną dla progresji guza w raku płaskonabłonkowym krtani.

Niniejsze badanie zostało przeprowadzone na dużej, reprezentatywnej grupie pacjentów z rakiem płaskonabłonkowym jamy ustnej. Zastosowano powtarzalne metody immunohistochemiczne z wykorzystaniem mikromacierzy tkankowych. Analizę poziomu białka przeprowadzono jednocześnie na komórkach nowotworu oraz limfocytach naciekających guz. Wymienione czynniki wpływają na rzetelność badania, a praca w pełni ukazuje związek badanych białek z czynnikami klinicznymi i patologicznymi oraz na ogólną przeżywalność chorych. Lokalizacja komórek odpornościowych i nowotworowych jest ważnym czynnikiem związanym z biomarkerami. Wyniki badania wykazały, że lokalizacja komórek z ekspresją PD-L1 odgrywa rolę czynnika prognostycznego i determinuje całkowite przeżycie pacjentów z rakiem płaskonabłonkowym jamy ustnej. Najważniejszym odkryciem było zależne od lokalizacji wartości związane z ekspresją PD-L1 w TCs i TILs. Nowotwory języka i dna jamy ustnej wykazywały mniejszą ekspresję niż nowotwory w pozostałych lokalizacjach. Gorszy całkowity czas przeżycia wiązał się z wyższą ekspresją PD-L1 na TCs w rakach języka i dna jamy ustnej niż w innych lokalizacjach. Z drugiej strony, wysoka ekspresja PD-L1 w TILs była powiązana z lepszym OS. Wykazano, że lokalizacja zmian jest bardzo ważna w ocenie rokowania przeżycia pacjenta i może być potencjalnym czynnikiem w praktyce klinicznej dla lepszego monitorowania pooperacyjnego chorych z rakiem płaskonabłonkowym jamy ustnej.

9. WNIOSKI

1 Wzrost poziomu białka PD-L1 na komórkach nowotworu i limfocytach naciekających guz był bardzo często obserwowany, natomiast ekspresja IL-33 na komórkach nowotworu i limfocytach naciekających guz była rzadko notowana w raku płaskonabłonkowym jamy ustnej.

2 Wysoka ekspresja białka PD-L1 na TILs była silnie skorelowana z brakiem przerzutów do węzłów chłonnych, a tym samym lepszym całkowitym przeżyciem we wszystkich lokalizacjach. Ekspresja białka PD-L1 na TCs miała wyraźny wpływ na przeżycie w nowotworach języka i dna jamy ustnej oraz w pozostałych lokalizacjach.

3 Ekspresja białka IL-33 nie miała znaczenia prognostycznego, ale obecność IL-33 na TCs była istotnie związana z lokalizacją guza.

4 Nie stwierdzono korelacji między ekspresją białek PD-L1 i IL-33.

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11. SPIS TABEL I RYCIN

12. OPUBLIKOWANE ARTYKUŁY WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ

1. **Adamski ŁJ,** Starzyńska A, Adamska P, Kunc M, Sakowicz-Burkiewicz M, Marvaso G, Alterio D, Korwat A, Jereczek-Fossa BA, Rafał Pęksa R. High PD-L1 expression on tumor cells indicates worse overall survival in advanced oral squamous cell carcinomas of the tongue and the floor of the mouth but not in other oral compartments. *Biomedicines* 2021, 9, 1132.
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High PD-L1 Expression on Tumor Cells Indicates Worse Overall Survival in Advanced Oral Squamous Cell Carcinomas of the Tongue and the Floor of the Mouth but Not in Other Oral Compartments

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Abstract: The markers of the tumor microenvironment (TME) are promising prognostic and predictive factors in oral squamous cell carcinoma (OSCC). The current study aims to analyze the immunohistochemical expression of programmed cell death-ligand 1 (PD-L1) and interleukin-33 (IL-33) in a cohort of 95 chemonaïve OSCCs. PD-L1 and IL-33 were assessed separately in tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs). High PD-L1 expression in TILs was associated with better overall survival (OS) in univariate analysis. Tumors localized in the floor of the oral cavity and tongue tended to have a lower percentage of PD-L1-positive TCs when compared to other locations. PD-L1 expression on TCs had no prognostic significance when the whole cohort was analyzed. However, along with the T descriptor (TNM 8th), it was included in the multivariable model predicting death in carcinomas of the floor of the oral cavity and tongue (HR = 2.51, 95% CI = 1.97–5.28). In other locations, only nodal status was identified as an independent prognostic factor in multivariate analysis (HR = 0.24, 95% CI = 0.08–0.70). Expression of IL-33 had no impact on survival, but it was differently expressed in various locations. In conclusion, the prognostic significance of PD-L1 in oral cancer depends on the tumor site and type of cell expressing immune checkpoint receptor (TCs vs. TILs).

Keywords: programmed cell death-ligand 1; PD-L1; interleukin 33; IL-33; oral squamous cell carcinoma; prognosis; immunohistochemistry

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts for 95% of all malignancies developing in the oral cavity. It predominantly affects males over 50 years of age and has a causal relationship with tobacco smoking and alcohol consumption [1]. Despite the substantial progress which has been made in OSCC treatment, overall survival (OS) has not improved significantly in the last few decades. The treatment for OSCC is multimodal, with surgery usually being the treatment of choice. Its clinical course mainly depends on the stage of

diagnosis, tumor location, and the feasibility of radical surgical resection. Carcinomas of the tongue and floor of the oral cavity are frequently locally advanced at the time of the first presentation and show a worse prognosis than other oral cancers. Nevertheless, other factors related to tumor biology and its immune microenvironment may also influence the prognosis or be the targets for personalized therapy [2–4].

The tumor microenvironment (TME) is a dynamic ecosystem consisting of cancer cell, stromal cells, blood vessels, extracellular matrix, and numerous types of immune cells which exhibit complex reciprocal interactions [5,6]. Cancer cells and immune cells may interact via programmed cell death ligand-1 (PD-L1/B7-H1/CD274) and its receptor programmed cell death-protein 1 (PD-1) [7]. PD-L1 expression by cancer cells is one of the mechanisms of immune response evasion, as it activates immune checkpoint protein PD-1 on cytotoxic CD8+ T lymphocytes and reduces their activity (a phenomenon which is called "exhaustion") [8]. Perplexingly, PD-L1 may be also expressed by immune cells, but the exact role of this process is not fully understood. In some cancers, PD-L1 expression on immune cells is associated with favorable outcomes, whereas in others it is a poor prognostic factor [9,10]. The PD-L1/PD-1 axis is a target of multiple drugs (immune checkpoint inhibitors), which have substantially improved survival, for example, in advanced non-small-cell lung carcinoma and melanoma [11–13]. To date, several studies aimed to assess the prognostic significance of the PD-L1/PD-1 axis in OSCC [2]. Combination of anti-PD-1 immunotherapy (pembrolizumab or nivolumab) with chemotherapy and radiation therapy improves outcomes in OSCC [14]. Conflicting data exists on the prognostic significance of PD-1/PD-L1 expression in OSCC. Predictive significance is also unclear, but both PD-L1 expressors and non-expressors benefit from anti-PD-L1 therapy [13,15].

Interleukin-33 (IL-33), a member of the IL-1 family, is a chromatin-associated cytokine released from the nucleus into extracellular space triggered by stress or necrosis, and serves as an endogenous danger signal (alarmin) [16]. Its role is to activate the immune system in response to tissue damage via interactions with its receptor, ST2, expressed by various immune cells. IL-33 is constitutively expressed by a variety of cells, including endothelial cells, fibroblasts, and epithelial cells, but it may be up-regulated in reaction to stresses. IL-33 plays a role in chronic inflammatory diseases like asthma and Crohn's disease, and bacterial, fungal, and parasitic infections [16–18]. Head and neck carcinomas overexpressing IL-33 in cancer cells and carcinoma-associated fibroblasts (CAFs) were characterized by higher invasiveness and worse outcomes [19]. Moreover, the IL-33/ST2 axis influences other elements of the TME, since it modifies the activity of T-helper lymphocytes, regulates the production of IL-4, IL-5, and IL-13, and angiogenesis [20,21]. Finally, IL-33 modulates PD-1/PD-L1 expression in the cancer microenvironment, and preliminary studies suggest that co-targeting of IL-33 and immune checkpoint receptors may improve the outcomes of immunotherapy [22]. The role of IL-33 in cancer is most likely context-dependent because it demonstrates either an anti-tumorigenesis effect (e.g., pancreatic cancer, ovarian, and colon cancer) or a pro-tumorigenesis effect (e.g., breast cancer, lung cancer) [20,23]. However, our knowledge about IL-33 expression in OSCC is scant [24,25].

In the current study, we aimed to evaluate PD-L1 and IL-33 expression in patients with OSCC in relation to clinical characteristics and survival.

2. Materials and Methods

2.1. Study Group

Medical records of 109 patients diagnosed with OSCC, treated at the University Clinical Center Medical University of Gdańsk, Poland, between 2007–2012 were analyzed. The inclusion criteria of this study included histopathologically confirmed OSCC and available treatment-naïve histopathological specimens (biopsy or resection). Patients without survival data were excluded (n = 14). Finally, ninety-five patients (n = 95) were included in the study. Basic demographic and clinicopathological data (age, gender, addictions, treatment methods, cancer localization, grading, staging, recurrence, death, follow-up, and survival rate) were collected. The staging in all cases was determined within one month after the

first presentation, and adjusted according to the American Joint Committee on Cancer (AJCC) 8th edition of the TNM classification for the sake of the current study. Patients' data were fully anonymized. The local Bioethical Committee of the Medical University of Gdańsk approved the protocol of the study (approval No NKBBN/59/2016).

2.2. Specimen Preparation and Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from tumor resection or, in the case of patients treated with neoadjuvant radiation or chemotherapy, from treatment-naïve biopsy (if applied) after the first presentation. Tissue microarrays (TMA) were prepared with a Manual Tissue Arrayer MTA 1 (Beecher Instruments Inc., Sun Prairie, WI, USA). Two representative cores, both of 0.4 cm diameter, were obtained from each case.

IHC was performed using the Ventana G11 system (CONFIRM™, Ventana Medical Systems, Tucson, AZ, USA). TMAs were stained with anti-PD-L1 antibody (rabbit monoclonal antibody, E1L3N, Cell Signaling, Danvers, MA, USA) and anti-IL-33 antibody (rabbit monoclonal antibody, MAB36252; Clone 1061A; R&D Systems, Inc., Bio-Techne, MN, USA).

The proportion of positive cells was established by calculating the number of stained tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs) divided by the total number of each type of cells. Two pathologists (AK and RP) experienced in PD-L1 expression evaluation assessed the stainings. When the interpretations differed, the pathologists made decisions by consensus. Only membranous PD-L1 expression was considered positive in TCs, whereas cytoplasmic and/or membranous reaction were considered positive in TILs. Only nuclear IL-33 expression was considered positive. Histologically normal tonsil was used for the positive control. For each patient, the results from two cores were used. The percentage of positively staining cells was estimated in each core and an average score was utilized in the further analyzes. The cut-off for high PD-L1 expression was established depending on the median of the percentage of positively staining cells as >10% in TCs and >20% in TILs (Figure 1A–D). IL33 was divided into two groups—no expression and positive expression (Figure 2A–D). Subsequently, we compared the agreement between two cores in the binary classification of PD-L1 and IL-33 by Cohen's kappa coefficient to assess the heterogeneity of markers expression.

2.3. Statistical Analysis

Statistical analysis was performed using the STATISTICA 13.3 (TIBCO, Palo Alto, CA, USA; licensed to the Medical University of Gdańsk) and R statistical environment [26]. Kaplan–Meier curves were plotted using the "survminer" and "ggsci" packages [27,28]. The associations between analyzed markers and clinicopathological characteristics were assessed by the Mann–Whitney U test for continuous variables. Categorical variables were compared by the chi-square test and Fisher's exact test when applicable. Cohen's kappa coefficient was calculated to assess the reproducibility across the two cores incorporated in the TMA. Kaplan–Meier curves were plotted to assess overall survival (OS) and compared by log-rank test. Hazard ratios were estimated with the Cox proportional hazard regression. The backward selection was employed to create a multivariable model predicting death and to eliminate non-significant variables at $p \leq 0.05$. All tests were considered statistically significant as $p \leq 0.05$.

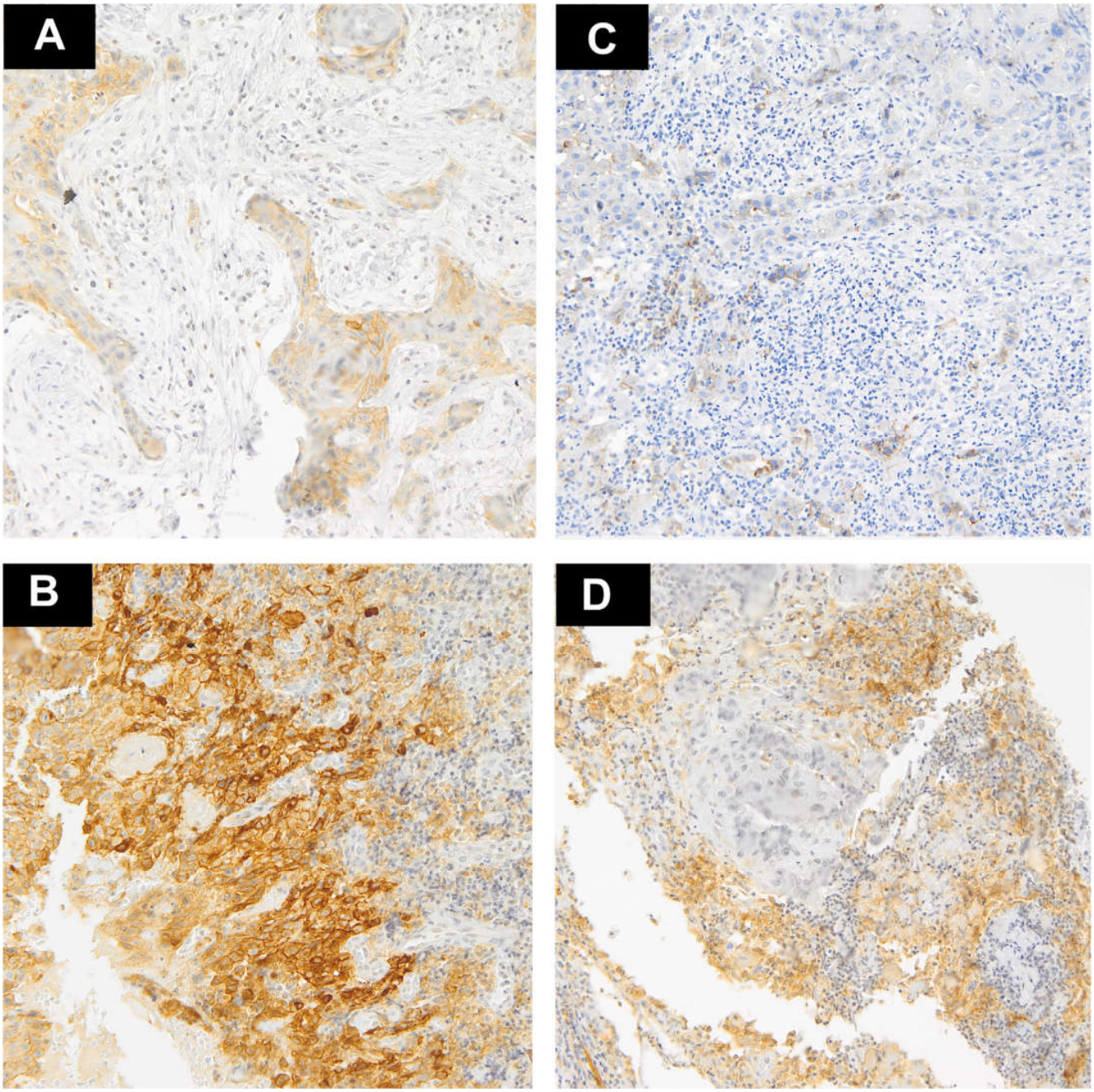

Figure 1. Representative examples of PD-L1 staining (magnification ×10). (**A**) A few tumor cells with weak expression of PD-L1; (**B**) Intense expression of PD-L1 in the majority tumor cells and weak staining in TILs at the tumor–stroma interface; (**C**) Very weak expression of PD-L1 on single lymphocytes and negative on cancer cells; (**D**) High expression of PD-L1 on TILs at the tumor–stroma interface and negative on tumor cells.

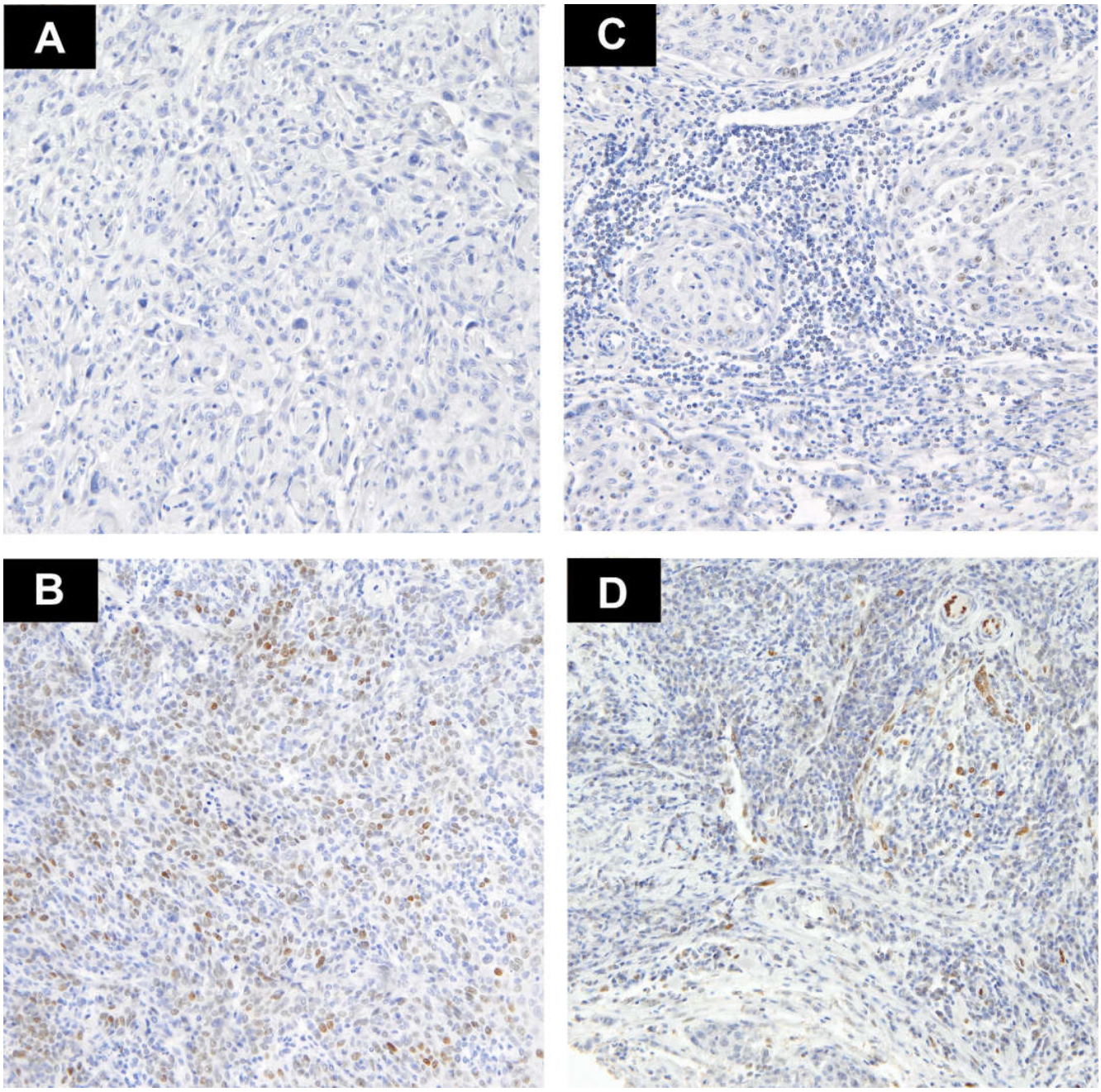

Figure 2. Representative examples of IL-33 staining (magnification ×10). (**A**) Tumor cells lacking IL-33 expression (no staining); (**B**) Positive nuclear staining with heterogeneous intensity in tumor cells; (**C**) TILs with negative IL-33 staining; (**D**) Weak positive nuclear staining in TILs and negative in TCs.

3. Results

3.1. PD-L1 Expression

Forty-four (46.31%) cases demonstrated positive expression of PD-L1 in > 10% of TCs. The mean percentage of positive cells was 21.88%, median 10%. Tumors localized in the floor of the oral cavity and tongue tended to have a lower percentage of PD-L1-positive TCs when compared to other locations ($p = 0.019$, Mann–Whitney U). There was a trend toward lower PD-L1 expression and the presence of nodal metastases ($p = 0.015$, Mann–Whitney U). Analogous findings were noted if PD-L1 was assessed as a binary variable (low/high expression). There was no association with gender, T stage, grade, history of smoking,

or alcohol abuse. The summary of clinicopathological features with relation to analyzed biomarkers is presented in Table 1.

Table 1. The summary of clinicopathological features with relation to PD-L1 expression on TCs and TILs (*p*—*p* value; *—statistically significant *p*). *p* values were calculated with chi square.

Parameters	Case Number n (%)	PD-L1 on TCs			PD-L1 on TILs		
		Low n (%)	High n (%)	*p*	Low n (%)	High n (%)	*p*
Gender							
Female	32 (33.68)	16 (16.84)	16 (16.84)	0.607	20 (21.05)	12 (12.63)	0.292
Male	63 (66.32)	35 (36.84)	28 (29.47)		46 (48.42)	17 (17.89)	
Smoking							
No	22 (30.14)	11 (15.07)	11 (15.07)	0.939	15 (20.55)	7 (10.27)	0.548
Yes	51 (69.86)	26 (35.62)	25 (34.25)		31 (42.47)	20 (27.40)	
Alcohol							
No	58 (80.56)	29 (40.28)	29 (40.28)	1.000	35 (48.61)	23 (31.94)	0.415
Yes	14 (19.44)	7 (9.72)	7 (9.72)		10 (13.70)	4 (5.56)	
Grade							
1	39 (42.05)	22 (23.16)	17 (17.89)	0.656	26 (27.37)	13 (13.68)	0.620
2–3	56 (58.95)	29 (30.53)	27 (28.42)		40 (42.11)	16 (16.84)	
Stage							
I–II	32 (42.67)	17 (22.08)	15 (15.79)	0.832	14 (18.18)	18 (18.18)	0.004 *
III–IV	45 (57.33)	25 (32.47)	20 (25.97)		34 (44.16)	11 (14.29)	
T							
1–2	42 (55.26)	23 (30.26)	18 (23.68)	0.874	23 (30.26)	19 (25.00)	0.157
3–4	34 (44.74)	18 (23.68)	16 (21.05)		24 (31.58)	10 (10.53)	
N							
0	39 (52.70)	17 (22.97)	22 (29.73)	0.097	18 (24.32)	21 (28.38)	0.006 *
1–3	35 (47.30)	22 (29.73)	13 (17.57)		27 (36.49)	8 (10.81)	
Location							
Tongue/ Floor of the oral cavity	55 (57.89)	35 (36.84)	20 (21.05)	0.022 *	37 (38.95)	18 (18.95)	0.584
Other	40 (42.11)	16 (16.84)	24 (25.26)		29 (30.53)	11 (11.58)	

Thirty-one (31.63%) cases displayed PD-L1 positivity in >20% of TILs (high expression). High PD-L1 expression on TILs was associated with the absence of lymph node metastases and lower stage. No association was found between the expression of PD-L1 in TILs and other analyzed clinicopathological variables.

The agreement between cores was moderate in terms of PD-L1 expression in TCs (Cohen's kappa = 0.645, 95% CI = 0.492–0.799), and fair in terms of PD-L1 expression in TILs (Cohen's kappa = 0.335, 95% CI = 0.126–0.545).

3.2. IL-33 Expression

Fifteen cases (15.79%) demonstrated IL-33 expression in >1% of TCs nuclei (mean 1.08%, median 0%, max. 30%). Cancers of the tongue and the floor of the oral activity expressed IL-33 less commonly ($p = 0.001$, chi-square). Stage 3–4 OSCCs tended to express IL-33 more commonly than lower stage tumors, but this finding had borderline statistical significance ($p = 0.057$, chi-square). No other clinicopathological variables showed association with IL-33 expressed by TCs.

Positive expression of IL-33 in TILs was observed in 18 cases (18.94%). Mean percentage was 0.5% (median 0%, maximum 7%). Expression of IL-33 in TILs was less

common in cancers of the tongue and the floor of the oral cavity (p = 0.055, chi-square), but no other association between IL-33 and clinicopathological variables was found (Supplementary Table S1).

Expression repeatability between IL-33 cores was substantial in terms of TCs (Cohen's kappa = 0.707, 95% CI = 0.487–0.927), and moderate in the case of TILs (Cohen's kappa = 0.493, 95% CI 0.235–0.752). No correlation was found between PD-L1 and IL-33 expression.

3.3. Survival Analysis

3.3.1. Whole Cohort

The mean follow-up was 3.83 years, the minimum was 24 days, and the maximum was 10.87 years. The 5-year OS was 36.65%. The univariate Cox's proportional hazard analysis (Table 2) demonstrated the association between survival and grade, stage, nodal metastases, and PD-L1 expression on TILs. Expression of IL-33 and PD-L1 in TCs had no impact on survival (Figure 3A for PD-L1, Supplementary Figures S1–S3 for IL-33). High PD-L1 expression in TILs was associated with better OS (HR = 0.475, 95% CI = 0.281–0.805; Figure 3B), but it was not retained in the multivariate Cox regression model predicting outcomes. Only the presence of nodal metastases was incorporated in the multivariable model predicting death.

Table 2. The univariate and multivariate Cox's proportional hazard analysis (HR—hazard ratio; 95% CI—confidence interval; p—p value; *—statistically significant p).

Feature	Univariate Cox's Proportional Hazard Analysis		Multivariate Cox Regression Model	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Gender (Male vs. Female)	1.026 (0.626–1.681)	0.919		
Smoking (No vs. Yes)	0.738 (0.392–1.389)	0.347		
Alcohol (No vs. Yes)	1.001 (0.485–2.065)	0.997		
Location (Tongue/floor of the oral cavity vs. other)	0.957 (0.596–1.537)	0.855		
Grade (2–3 vs. 1)	1.767 (1.074–2.906)	0.025 *		
Stage (1–2 vs. 3–4)	0.333 (0.185–0.598)	<0.001 *		
T (1–2 vs. 3–4)	0.383 (0.221–0.664)	<0.001 *		
N (0 vs. 1–3)	0.343 (0.196–0.600)	<0.001 *	0.345 (0.193–0.617)	<0.001
PD-L1 TCs (high vs. low)	0.991 (0.618–1.589)	0.971		
PD-L1 TILs (high vs. low)	0.525 (0.306–0.902)	0.012 *		
IL-33 TCs (positive vs. negative)	0.837 (0.428–1.637)	0.603		
IL-33 TILs (positive vs. negative)	1.036 (0.566–1.894)	0.909		

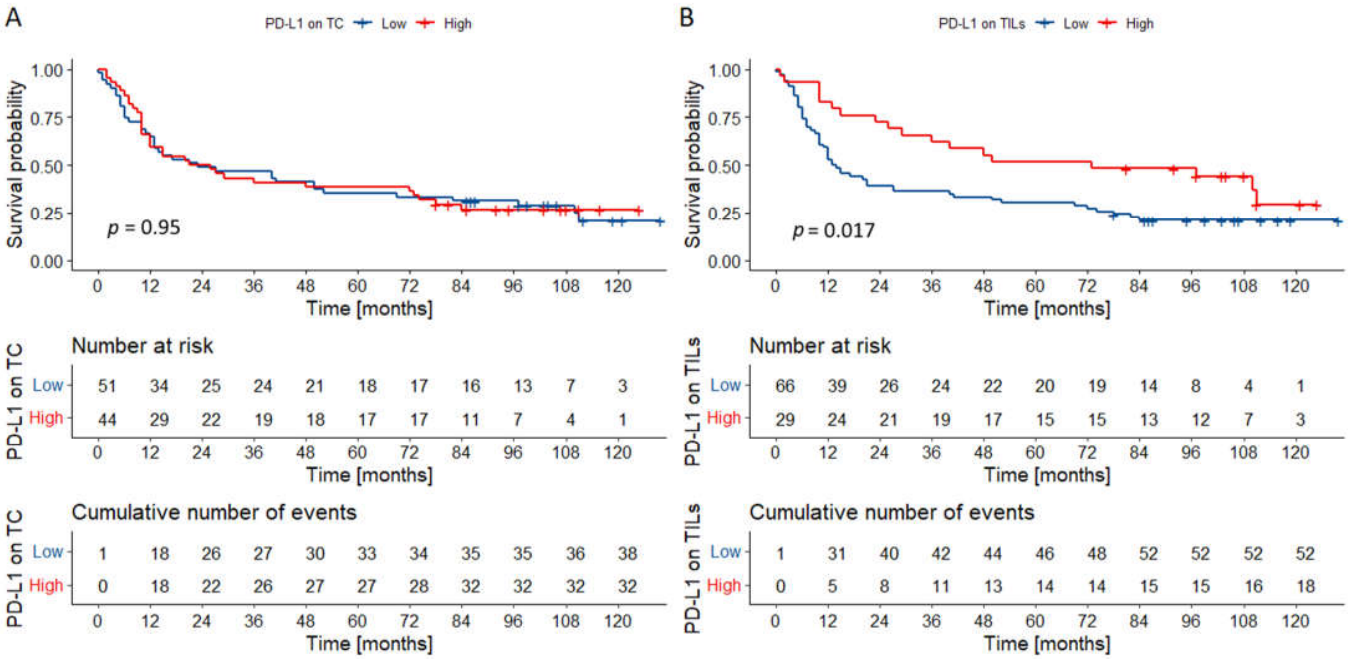

Figure 3. Kaplan–Meier curves for overall survival according to PD-L1 expression on TCs (**A**), and TILs (**B**). PD-L1 expression on TCs had no impact on the outcomes in the whole cohort. High PD-L1 expression on TILs was associated with superior overall survival. *p* values were calculated with the log-rank test. Abbreviations: TC—tumor cells; TILs—tumor-infiltrating lymphocytes.

3.3.2. Prognostic Significance of PD-L1 and IL-33 Expression in Various Locations

Due to the significant differences in PD-L1 expression in cancers of the tongue and floor of the oral cavity, we decided to create separate multivariable Cox's regression models that predict outcomes and take into consideration cancer location.

In the group of cancers of the tongue and the floor of the oral cavity, two variables were incorporated in the final model: T category and PD-L1 expression on TCs (Table 3). Interestingly, in univariate analysis, PD-L1 expression on TCs and TILs had a statistically borderline impact on survival (Figure 4). Especially poor outcomes were observed in the group of T3–4 tumors highly expressing PD-L1 on TCs (Figure 5).

Table 3. The multivariate Cox's regression models (HR—hazard ratio; 95% CI—confidence interval; *p*—*p* value; *—statistically significant *p*).

Multivariate Cox Regression Model		
Feature	**HR (95% CI)**	***p***
Tongue/Floor of the oral cavity		
T (1–2 vs. 3–4)	0.229 (0.101–0.518)	<0.001 *
PD-L1 TCs (high vs. low)	2.514 (1.977–5.282)	0.014 *
Other locations		
N (0 vs. 1–3)	0.239 (0.081–0.699)	0.008 *

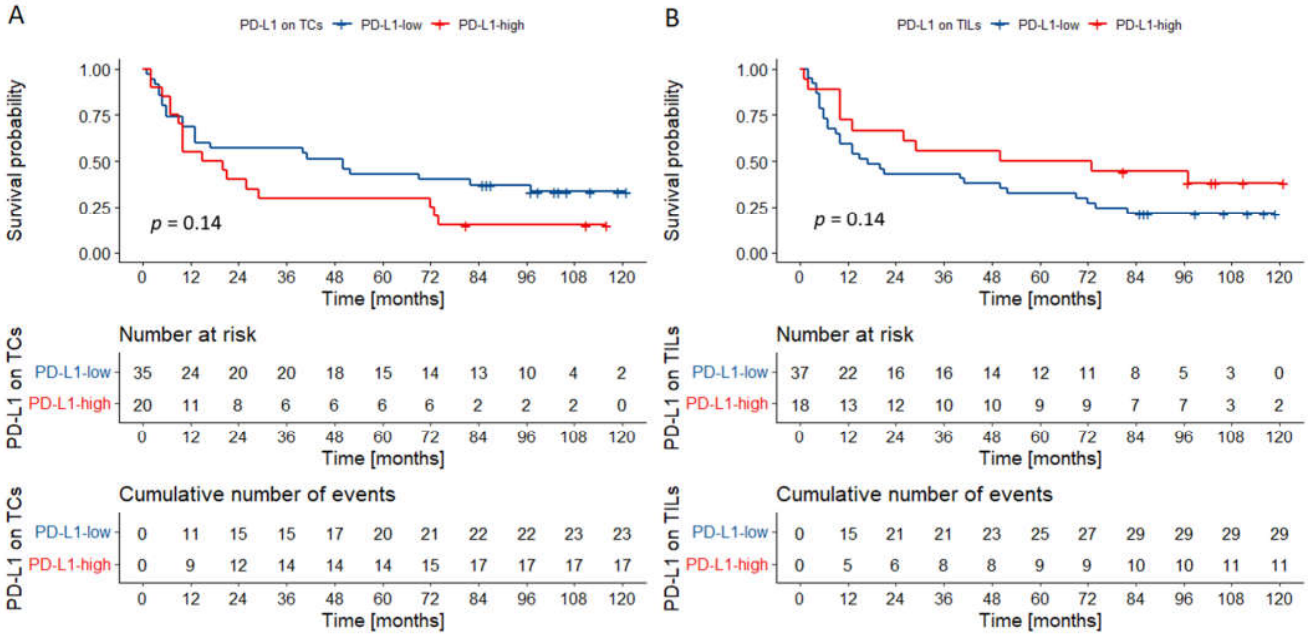

Figure 4. Overall survival probability curve according to PD-L1 expression on TCs (**A**) and TILs (**B**) in OSCCs of the tongue/floor of the oral cavity (**A**,**B**). There was a trend towards worse overall survival in cancers of the tongue/floor of the oral cavity with high PD-L1 expression on TCs. An opposite trend was observed for PD-L1 expression on TILs. *p* values were calculated with the log-rank test. Abbreviations: TCs—tumor cells; TILs—tumor infiltrating lymphocytes; OSCC—oral squamous cell carcinoma.

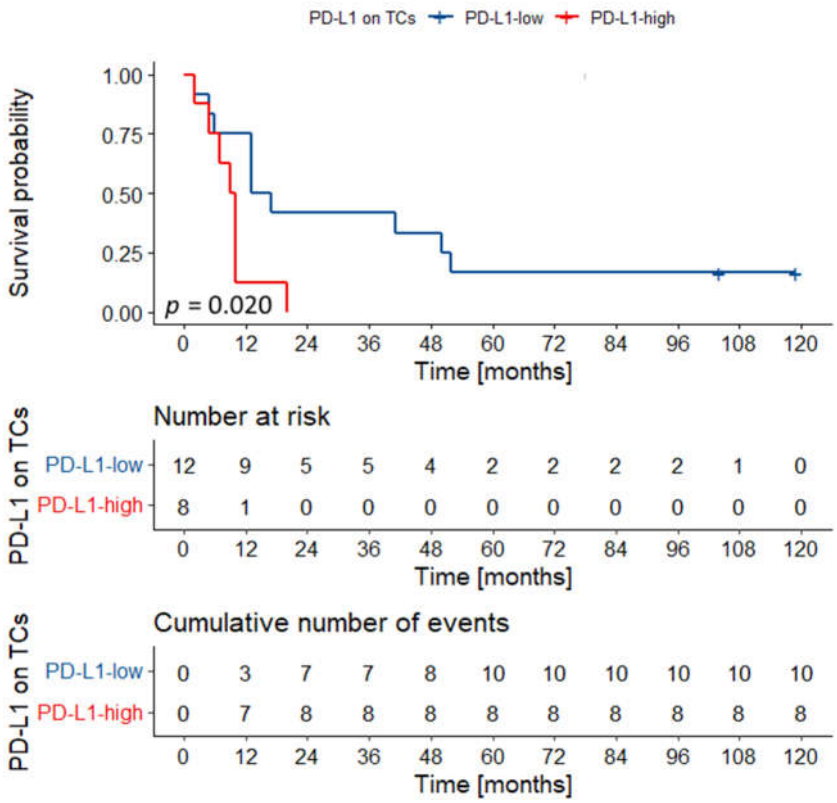

Figure 5. Overall survival probability curve according to PD-L1 expression on TCs in T3–T4 tumors of the tongue/floor of the oral cavity. Tumors with high PD-L1 expression on TCs had dismal outcomes. *p* value was calculated with the log-rank test. Abbreviations: TCs—tumor cells.

In cancers located in other parts of the oral cavity, only the presence of nodal metastases was incorporated in the multivariable model. Importantly, in univariate analysis, PD-L1 expression on TILs was associated with better outcomes (Figure 6).

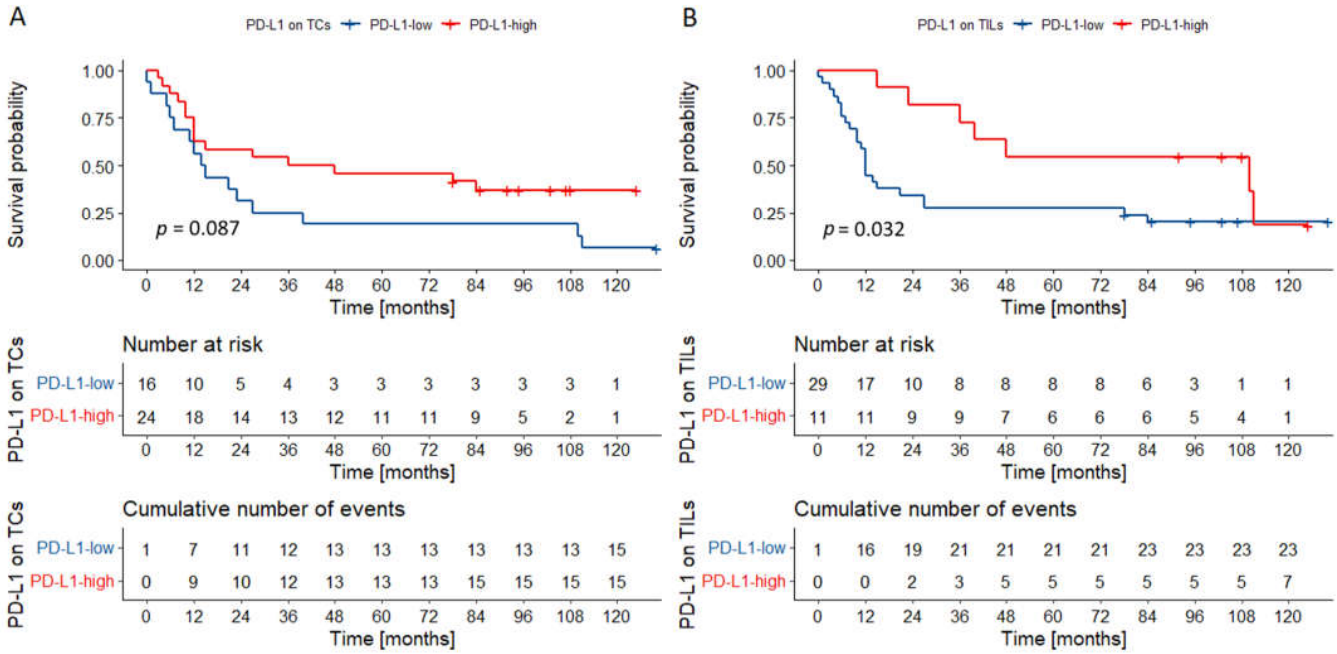

Figure 6. Overall survival probability curves according to PD-L1 expression on TCs (**A**) and TILs (**B**) in other oral compartments. There was a trend toward better survival in tumors characterized by high PD-L1 expression on TCs. Tumors rich in PD-L1-positive TILs had superior outcomes. *p* values were calculated with the log-rank test. Abbreviations: TCs—tumor cells; TILs—tumor infiltrating lymphocytes.

4. Discussion

The oral cavity is in constant contact with the external environment. There are numerous reactions here that are designed to protect the body against harmful factors. The importance of the immune tumor microenvironment and tumor immunology in the prognosis of patients with OSCCs and other head and neck malignancies is becoming increasingly recognized [2,3,29,30]. This is the first study to co-analyze PD-L1 and IL-33 protein expression on TCs and TILs in OSCC. We demonstrated that the level of PD-L1 expression on TCs varies depending on its location—cancers of the tongue and the floor of the oral cavity show lower expression than cancers of other parts. The expression of PD-L1 on TCs was not related to OS in the entire cohort. However, the expression of PD-L1 on TCs appears to have opposite effects in cancers of different locations, which "canceled out" when analyzing the entire cohort. Higher PD-L1 expression on TCs in the carcinomas of the tongue/floor of the oral cavity was associated with a worse OS, especially in cancers of higher T category in TNM. In other locations, higher PD-L1 expression on TCs was associated with a better prognosis (a trend, but not statistically significant). On the other hand, higher PD-L1 expression on TILs was associated with a lower frequency of nodal metastases. Moreover, it was associated with longer OS in the univariate analysis, but this effect was not maintained in the multivariate analysis. Most likely, this is due to the very strong correlation of PD-L1 on TILs and nodal metastases. The latter was identified as the single most important prognostic factor in the whole cohort. These findings are supported by studies on large populations, which demonstrated that the number of metastatic lymph nodes and its characteristics (e.g., the presence of extranodal extension) are critical predictors of survival in OSCC [31,32].

In other works concerning OSCC, PD-L1 expression on TCs was observed with variable frequency ranging from approximately 10 to 90% of cases [33–66]. The studies differed in the size of the study group, selection of patients, antibody clones used, and the way of assessing PD-L1 expression; the two latter factors in particular significantly influence the final results of the study. In the Supplementary Table S2, we briefly present the previous studies analyzing PD-L1 expression in OSCC with the emphasis on methodology and prognostic effects.

Most studies to date, similarly to ours, are based on the heterogeneous cohorts of tumors located throughout the oral cavity, with a few exceptions focused on certain oral compartments, e.g., squamous cell carcinoma of the tongue. Multiple studies assessing survival in OSCCs demonstrated that high PD-L1 expression on TCs is associated with worse outcomes. Strati et al. [67] demonstrated that PD-L1 overexpression on circulating TCs was associated with inferior progression-free survival and OS in head and neck cancers. Nevertheless, a few studies showed contrary results or no association between PD-L1 expression and survival. The results of our study suggest that these discrepancies may originate from skewed distribution of cancer location in analyzed cohorts, as PD-L1 expression on TCs seems to have different prognostic effects in various compartments of the oral cavity. The site-dependent differences in the TME composition of head and neck carcinomas were previously reported by Green et al. [68], who observed a higher prevalence of TILs in oropharyngeal cancers compared to other locations. Interestingly, PD-L1 is physiologically expressed on the masticatory mucosa of the oral cavity, whereas other epithelia do not constitutively express PD-L1 [69]. Additionally, oral compartments represent ecological niches inhabited by a variety of microbiota modulating the local immune microenvironment [70,71]. For instance, *Porphyromonas gingivalis* induces the expression of PD-L1 in OSCC cells in vitro [53]. Finally, the immune landscape of HPV- and carcinogen-driven head and neck carcinomas differ in some aspects [72]. All these factors may contribute to distinct immune characteristics of OSCCs of various sites.

However, the prognostic significance of PD-L1 expression in TCs may also depend on other factors, especially the TME context in certain parts of the oral cavity [73]. Takahashi et al. [42] demonstrated that patients with high PD-L1 expression and abundant CD4+ T-cells have better outcomes than those with low CD4+ T-cell infiltration. Other research showed that high infiltration by CD4+ and CD8+ and high CD8+/FOXP3+ ratio lymphocytes were associated with positive expression of PD-L1 on TCs [33]. Other factors which influence PD-L1 expression in OSCC include gender, since some studies showed more PD-L1-negative tumors in males than in females [37,39]. Hanna et al. [44] reported that PD-L1 expression was associated with better outcomes in young females with OSCC. In another study, PD-L1 expression on TCs was more common in non-smokers and non-drinkers [52].

Even more perplexing is the role of PD-L1 expression on immune cells in OSCC. Previous translational research demonstrated that PD-L1-positive macrophages induce anergy in CD4+ and CD8+ T-cells in OSCC TME [56,57]. However, the current study demonstrates the more favorable prognosis of OSCC infiltrated by the high number of PD-L1-positive TILs. This effect was strongly correlated with the absence of nodal metastases. These results are consistent with the study by Kim et al. [10] which analyzed a large cohort of head and neck squamous cell carcinomas (including 204/402 oral cancers), and reported that PD-L1 expression on TILs, but not on TCs, was a favorable prognostic factor. Similar associations were noted in laryngeal cancer [74]. Better outcomes and lack of nodal metastases in tumors rich in PD-L1 positive TILs in OSCCs may be related to preexistent anti-tumor adaptive immune response [75].

In our study, IL-33 was rarely expressed in OSCC and had no significant impact on patients' survival. In the only study so far analyzing IL-33 in oral cancer, Ishikawa et al. [24] evaluated IL-33 expression in squamous cell carcinoma of the tongue [24]. The authors showed that high IL-33 expression in tumor cells was associated with local and nodal recurrence. They used a different antibody clone (IL-33, MBS150331, rabbit polyclonal antibody, Medical & Biological Laboratories, Nagoya, Japan) and immunostaining method

analysis, which may explain the discrepancies [24]. IL-33 is able to increase PD-1 and PD-L1 expression at the surface of CD8+ T lymphocytes and cancer cells, respectively [22]. The process is most likely driven via enhanced T cell production of IFN-γ. However, we did not find any association between the expression of PD-L1 and IL-33 in our cohort.

Unfortunately, our study has several limitations. The cohort size is suboptimal and the number of cases representing various compartments is low. Thus, it is impossible to draw definite conclusions regarding the survival analysis.

5. Conclusions

PD-L1 was commonly expressed in OSCC by TCs and TILs in our cohort. However, OSCC immunobiology and prognostic significance of PD-L1 expression vary depending on tumor location. High PD-L1 expression on TILs was strongly correlated with the lack of nodal metastases and, thus, better OS in all locations. On the other hand, PD-L1 expression on TCs seems to have a distinct impact on survival in cancers of the tongue and floor of the oral cavity and other locations. Our findings have the potential to be applied in clinical practice for better post-operative OSCC monitoring. Nevertheless, it is unknown if this factor may influence the response to immune checkpoint blockade. IL-33 expression was rarely observed and had no prognostic significance but its expression on TCs was significantly associated with tumor location. It supports site-specific variations in TME of oral cancer. Thus, future research on the immune landscape of OSCC and its responsiveness to immune therapy should focus on the analysis of cancers from distinct compartments of the oral cavity.

Supplementary Materials: Supplementary materials can be found at https://www.mdpi.com/article/10.3390/biomedicines9091132/s1, Figure S1: Overall survival probability curves according to IL-33 expression on TILs (A) and TCs (B) in the whole cohort, Figure S2: Overall survival probability curves according to IL-33 expression on TILs (A) and TCs (B) in cancers of the tongue/floor of the oral cavity, Figure S3: Overall survival probability curves according to IL-33 expression on TILs (A) and TCs (B) in cancers of other oral compartments, Table S1: The summary of clinicopathological features with relation to IL-33 expression on TCs and TILs, Table S2: The summary of studies investigating immunohistochemical expression of PD-L1 in OSCC.

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Abbreviations

AJCC	American Joint Committee on Cancer
CAFs	carcinoma-associated fibroblasts
CI	confidence interval
FFPE	formalin-fixed paraffin-embedded
HR	hazard ratio
IHC	immunohistochemistry
IL-33	interleukin 33
IFN-γ	interferon gamma
M	lymph nodes
N	metastasis
OS	overall survival
OSCC	oral squamous cell carcinoma
p	*p* value
PD-L1	programmed cell death-ligand 1
ST2	membrane receptor soluble interleukin 1 receptor-like 1
T	tumor
TCs	tumor cells
TILs	tumor infiltrating lymphocytes
TMA	tissue microarray
TME	tumor microenviroment
TNM	tumor, lymph nodes, metastasis—TNM Classification of Malignant Tumors

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Original paper

The B7 family molecules in oral squamous cell carcinoma: a systematic review. Part I: B7-H1 (PD-L1) and B7-DC (PD-L2)

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) is the most common cancerous lesion in the oral cavity. During recent years, no significant reduction in the survival rate has been observed.
Aim: To systematically review the literature and to summarise correlations between B7 family proteins and prognosis in OSCC.
Material and methods: A systematic review of the literature about B7-H1 (PD-L1) and B7-DC (PD-L2) was carried out, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Thirty-six articles published before 22 May 2020 were included in the systematic review.
Results: The biggest study group consisted of 305 patients and the smallest – 10 patients. PD-L1 proved to be a prognostic factor in patients with OSCC. Immunohistochemistry was the most commonly used diagnostic method.
Conclusions: Any mutations in the gene encoding PD-L1 and quantitative or functional changes in the status of PD-L1 may be important in the prognosis of OSCC.

Key words: squamous cell carcinoma, oral cavity, B7 antigens.

Introduction

Ninety-five percent of oral neoplasms are diagnosed as squamous cell carcinoma (oral squamous cell carcinoma – OSCC). In 2017, about 390,000 new cases around the world were diagnosed (lip and oral cavity). Men suffer more often than women, especially in the sixth decade of life [1–4]. This type of cancer mainly affects people living in South-Central Asia, Central and Eastern Europe and in Malaysia [1, 2]. Smoking, betel chewing, alcohol abuse and HPV infection are the main risk factors. There is a systematic increase in the prevalence among women and patients under 50 years of age [3, 4].

In a properly functioning organism, damaged cells undergo apoptosis, a process in which various elements of the immune system participate. The main role is played by T and NK (natural killers) lymphocytes. In tumour pathology, cells gain unlimited ability to divide and survive as a result of changes in their genetic material. Lymphocyte function is the result of a balance between up-regulation (stimulation) and down-regulation (inhibition). Some types of cancers exhibit immunogenicity, i.e. the ability to induce an anti-cancer reaction. There are several molecular mechanisms involved in the regulation of the microenvironment of cancer cells and in the protection against the attack of the immune system [5]. Activation of T cells requires two signals. The first one is T cell receptor (TCR) activation – the major histocompatibility complex (MHC) on antigen-presenting cells (APC).

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The second signal is based on co-stimulatory molecules such as the B7-CD28 pathway [6].

The B7/CD28 family receptors are found on cells of the immune system. These are tumour infiltrating lymphocytes (TILs). Ligands (B7 proteins) are expressed on APC cells, immune cells, epithelial cells, osteoblasts, fibroblasts and others. In addition, the presence of ligands on tumour cells was detected [7]. The B7 family includes proteins and ligands as shown in Figure 1. The B7/CD28 pathway influences the regulation of the immune response by limiting both time and strength of the inflammatory response. Although the co-stimulation mechanism of the B7/CD28 pathway is not known, monoclonal antibodies are currently used in targeted therapies of malignant tumours, autoimmune and infectious diseases [7–9].

Aim

The aim of this paper was to collect and review the B7 family proteins as prognostic factors in OSCC and to describe their role in aggressive disease progression. This particular study includes B7-H1 (PD-L1) and B7-DC (PD-L2) proteins.

Material and methods

For this review, a systematic search of the literature was conducted in the PubMed, Web of Science, Scopus, Embase, Cochrane Library, and Google Scholar databases to identify papers containing data about the B7 family proteins in OSCC. The PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) were used [10].

PICO for study characteristics was used (P – patient/population/problem; I – intervention/exposure; C – comparison; O – outcome):

P: at least 10 patients with primary oral squamous cell carcinoma;
I: protein expression evaluation;
C: not required;
O: B7 family proteins as prognostic factors in OSCC – only B7-H1 (PD-L1) and B7-DC (PD-L2) proteins.

Inclusion criteria for papers were as follows: at least 10 patients with primary oral squamous cell carcinoma, B7 protein expression evaluation, study conducted only on humans, in the English language, prospective and retrospective studies, clinical studies and immunohistochemical evaluation of B7 protein. Articles were excluded if specimens derived from OSCC recurrences (not primary tumours), was conducted on cell lines, was not conducted on humans, the study group consisted of less than 10 patients, non-B7 family protein expression was evaluated or the study was not in English. Duplicate records as well as letters and papers that did not contain significant information were also excluded.

A retrospective analysis of articles on the B7 family proteins as risk factors in OSCC published from 2011 to 22 May 2020, was performed. Key words: "B7 family and oral cancer/OSCC/oral squamous cell carcinoma", "PD-1/PD-L1/PD-L2 pathway and oral cancer/OSCC/oral squamous cell carcinoma", "B7-H1/PD-L1/CD274/PDCD1LG1/B7H1/B7-H/PDCD1L1/PDCD1LG1/PDL1 and oral cancer/OSCC/oral squamous cell carcinoma", "B7-DC/CD273/PDCD1LG2/B7DC/Btdc/PDCD1L2/PDL2/bA574F11.2 and oral cancer/OSCC/oral squamous cell carcinoma", "PD1 signal transduction and oral cancer/OSCC/oral squamous cell carcinoma" were used. Articles were screened and sorted based on titles and abstracts. Then articles were evaluated for eligibility. Data extracted from those records were analysed in detail. The following pieces of information were collected: total patient number, occurrence of B7 family alterations in OSCC, correlations with age, gender, grading, primary tumour size (T stage), nodal metastases (N stage), staging, prognostic significance and diagnostic methods (immunohistochemistry and other methods). For randomized studies, the Cochrane Collaboration tool [11] and the methodological index for non-randomized studies (MINORS) were used [12]. The ideal global score for non-comparative studies is 16 and for comparative is 24 [12].

Statistical analysis

Statistical analysis was carried out using the Statistica 13.3 (StatSoft Inc. Tulsa, United States), licensed by the Gdansk Medical University.

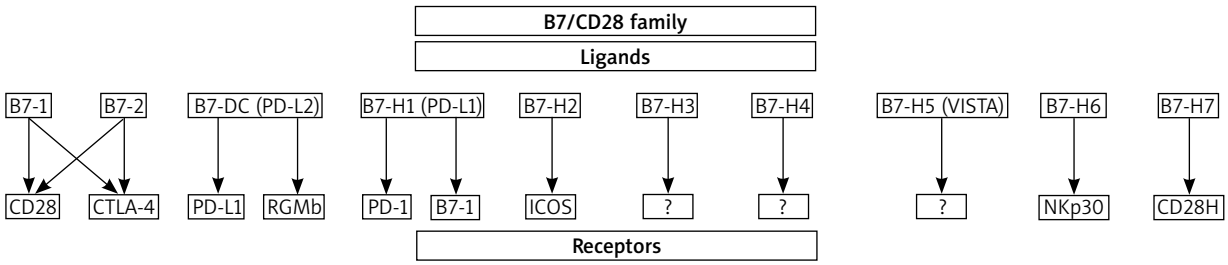

Figure 1. The B7 family ligands and receptors (? - unknown receptor)

Results

In the first step of selection 923 references were identified. 533 records were selected after exclusion of the duplicates. Eventually, 36 articles were included in the systematic review (Figure 2) [13–48]. Three studies involved PD-L2 while 35 articles were on PD-L1. The first identified study was published in 2011. Table 1 shows the articles included in the analysis [13–48].

All presented papers were observational and cohort-based studies (Tables 2, 3). There were no randomized controlled trials. 86.11% were retrospective (n = 31), 11.11% of the studies were prospective (n = 4) and 2.78% were retro-prospective (n = 1).

The biggest study group consisted of 305 patients and the smallest – 10 patients. In total, 3170 patients (excluding duplicates) were analysed in the studies. The occurrence of protein expression was as follows: PD-L1 – 18.4–100% and PD-L2 – 23.8–100%. PD-L1 protein was associated with gender [16, 18, 35, 41, 44], grading [19, 48], primary tumour size (T stage) [21, 29, 30] and metastases in lymph nodes [17, 25, 39, 48]. Staging was correlated

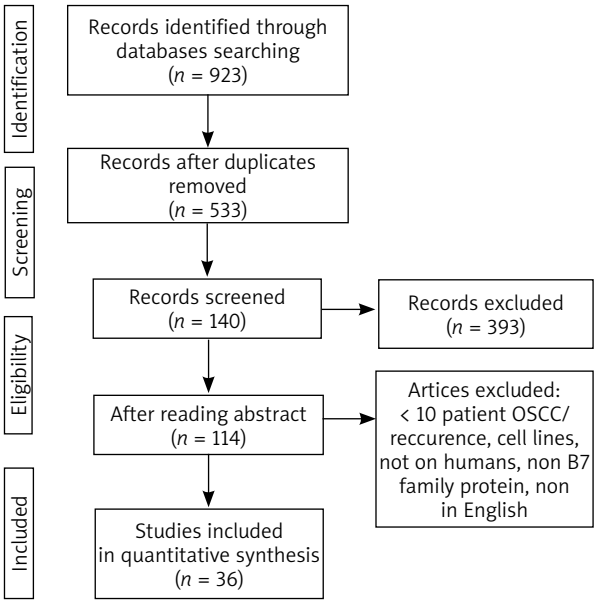

Figure 2. PRISMA flow diagram of study selection

Table 1. B7 family genetic alterations in oral squamous cell carcinomas: a review of current studies

No.	Reference	Study type	Total patient number	Occurrence of protein expression n patients (%)	Correlations							Methods
					Age	Gender	Grading	Primary tumour size (T stage)	Nodal metastases (N stage)	Staging	Prognostic significance	
B7-H1 (PD-L1):												
1	Cui *et al.*, 2020 [13]	O, R, C	34	No data	NE	NE	NE	NE	NE	NE	NE	IHC
2	Meehan *et al.*, 2020 [14]	O, R, C	67	No data	NE	NE	NE	NE	NE	NE	NS	IHC
3	Quan *et al.*, 2020 [15]	O, R, C	159	No data	NE	NE	NE	NE	NE	NE	OS – NS p = 0.742	IHC
4	Wilms *et al.*, 2020 [16]	O, R-P, C	101	80 (79.2%)	NS p = 0.494	S p = 0.019	NE	NS p = 0.929	NS p = 0.286	NS p = 0.888	OS – S p = 0.021 DFS – S p = 0.020	IHC
5	Zhao *et al.*, 2020 [17]	O, R, C	46	30 (65.2%)	NS p = 0.829	NS p = 0.956	NS p = 0.806	NS p = 0.052	S p = 0.009	S p = 0.011	NE	IHC
6	Ahmadi *et al.*, 2019 [18]	O, R, C	255	70 (27.5%)	NS p = 0.610	S p = 0.005	NS p = 0.760	NS p = 0.260	NS p = 0.660	NE	OS – NS p = 0.482 DSS – NS p = 0.864 DFS – NS p = 0.731	IHC
7	Chen *et al.*, 2019 [19]	O, P, C	41	40 (97.6%)	NS p = 0.088	NS p = 0.857	S p = 0.010	NS p = 0.9414	NS p = 0.147	NE	OS – NS p = 0.696 DFS – NS p = 0.210	IHC
8	de Vincente *et al.*, 2019 [20]	O, R, C	125	4 (3.2%)	NE	NS p = 0.530	NS p = 1.000	NS p = 0.580	NS p = 0.570	NS p = 0.350	DFS – S p = 0.030	IHC
9	Kouketsu *et al.*, 2019 [21]	O, R, C	106	73 (68.9%)	NS p = 0.999	NS p = 511	NE	S p = 0.018	NS p = 0.472	S p = 0.010	OS – NS	IHC

Table 1. Cont.

No.	Reference	Study type	Total patient number	Occurrence of protein expression *n* patients (%)	Correlations							Methods
					Age	Gender	Grading	Primary tumour size (T stage)	Nodal metastases (N stage)	Staging	Prognostic significance	
10	Takahashi *et al.*, 2019 [22]	O, R, C	77	46 (60%)	NS $p = 0.880$	NS $p = 0.360$	NS $p = 0.420$	NS $p = 0.750$	NS $p = 0.790$	NS $p = 0.580$	PFS – NS $p = 0.540$ OS – NS $p = 0.920$	IHC
11	Tojyo *et al.*, 2019 [23]	O, R, C	48	44 (91.7%)	NS $p = 1.000$	NS $p = 1.000$	NS $p = 0.540$	NS $p = 0.520$	NS $p = 1.000$	NE	DFS – NS $p = 0.185$	IHC
12	Hanna *et al.*, 2018 [24]	O, R, C	81 (32 females)	28 females (87%)	NE	NE	NE	NE	NE	NE	Female OS – S $p < 0.001$	IHC
13	Maruse *et al.*, 2018 [25]	O, R, C	97	63 (64.9%)	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	S $p = 0.050$	NS $p > 0.050$	NS $p > 0.050$	IHC
14	Stasikowska-Kanicka *et al.*, 2018 [26]	O, R, C	78	62 (79%)	NE	NE	NE	NE	NE	NE	PP – S $p < 0.011$	IHC
15	Stasikowska-Kanicka *et al.*, 2018 [27]	O, R, C	70	67 (96%)	NE	NE	NE	NE	NE	NE	NE	IHC
16	Udeabor *et al.*, 2018 [28]	O, R, C	20	No data	NE	NE	NE	NE	NE	NE	NE	IHC
17	Wirsing *et al.*, 2018 [29]	O, R, C	75	45 (60%)	NE	NE	NE	S $p = 0.024$	NS	NE	DSD – NS $p = 0.207$	IHC
18	Ahn *et al.*, 2017 [30]	O, R, C	68	45 (66%)	NS $p > 0.050$	NS $p > 0.050$	NE	S $p = 0.002$	NS $p = 0.648$	S $p = 0.010$	DFS – NS $p = 0.070$ OS – S $p = 0.039$	IHC
19	Feng *et al.*, 2017 [31]	O, R, C	119	No data	NE	NE	NE	NE	NE	NE	OS – S $p = 0.007$	IHC
20	Foy *et al.*, 2017 [32]	O, R, C	44	No data	NE	NE	NE	NE	NE	NE	NE	IHC
21	Groeger *et al.*, 2017 [33]	O, P, C	15	15 (100%)	NE	NE	NE	NE	NE	NE	NE	IHC
22	Hirai *et al.*, 2017 [34]	O, P, C	24	No data	NE	NE	NS 0.145	NS $p = 0.873$	NS $p = 0.542$	NE	NE	IHC
23	Kogashiwa *et al.*, 2017 [35]	O, R, C	84	44 (52%)	NS $p = 0.492$	S $p = 0.010$	NE	NS $p = 0.613$	NS $p = 0.734$	NS $p = 0.235$	OS – S $p = 0.006$ PFS – S $p = 0.024$	IHC
24	Kubota *et al.*, 2017 [36]	O, R, C	46	No data	NE	NE	NE	NE	NE	NE	NE	IHC
25	Mattox *et al.*, 2017 [37]	O, R, C	53	39 (73%)	NE	NE	NE	NE	NE	NE	OS – NS $p = 0.830$	IHC
26	Takakura *et al.*, 2017 [38]	O, R, C	10 (patients without chemotherapy)	8 (80%)	NE	NE	NE	NE	NE	NE	NE	IHC
27	Troeltzsch *et al.*, 2017 [39]	O, R, C	88	26 (29%)	NS $p = 0.349$	NS $p = 0.579$	NS $p = 0.157$	NS $p = 0.831$	S $p = 0.039$	NE	DSS – NS $p = 0.937$	IHC

Table 1. Cont.

No.	Reference	Study type	Total patient number	Occurrence of protein expression *n* patients (%)	Correlations							Methods
					Age	Gender	Grading	Primary tumour size (T stage)	Nodal metastases (N stage)	Staging	Prognostic significance	
28	Weber *et al.*, 2017 [40]	O, R, C	45	35/43 (1.:81.4% increased PD-L1_4); 32/43 (2.:74.4% increased PD-L1_2)	NE	NE	1. G1 vs. G3 – S $p = 0.020$ 2. G1 vs. G3 – S $p = 0.010$	1. NS $p = 0.370$ 2. NS $p = 0.487$	1. S $p < 0.002$ 2. S $p = 0.003$	NS $p > 0.05$	NE	IHC
29	Satgunaseelan *et al.*, 2016 [41]	O, R, C	217	40 (18.4%)	NS $p = 0.493$	S $p = 0.013$	NS $p = 0.060$	NS $p = 0.550$	NS $p = 0.900$	NE	DSS – NS $p = 0.960$ DFS – NS $p = 0.820$ OS – NS $p = 0.930$	IHC
30	Straub *et al.*, 2016 [42]	O, R, C	80	36 (45%)	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	OS – S $p = 0.010$ RFS $p = 0.050$	IHC
31	Chen *et al.*, 2015 [43]	O, R, C	218	139 (64%)	NE	NE	NE	NE	NE	NE	DFS – NS $p = 0.020$ OS – NS $p = 0.110$	IHC
32	Lin *et al.*, 2015 [44]	O, R, C	305	134 (44%)	NS $p = 0.124$	S $p = 0.006$	NS $p = 0.326$	NS $p = 0.316$	NS $p = 0.736$	NS $p = 0.804$	OS – NS $p = 0.083$	IHC
33	Oliveira-Costa *et al.*, 2015 [45]	O, R, C	142	47/97 (49%)	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	NS $p > 0.050$	DSS – S $p = 0.044$	IHC
34	Cho *et al.*, 2011 [46]	O, R, C	45	39 (87%)	NS $p = 0.787$	NS $p = 0.745$	NS $p = 0.158$	NS $p = 0.393$	NS $p = 0.433$	NS $p = 0.736$	OS – NS $p = 0.501$	IHC
35	Malaspina *et al.*, 2011 [47]	O, R, C	39	No data	NE	NE	NE	NE	NE	NE	NE	IHC
B7-DC (PD-L2):												
1	Weber *et al.*, 2019 [48]	O, P, C	48	28/36 (77.8%)	NE	NE	NS $p = 0.130$	NS $p = 0.805$	NS $p = 0.960$	NE	NS $p = 0.400$	RT-qPCR
2	Groeger *et al.*, 2017 [33]	O, P, C	15	15 (100%)	NE	NE	NE	NE	NE	NE	NE	IHC
3	Kogashiwa *et al.*, 2017 [35]	O, R, C	84	20 (23.8%)	NS $p = 0.792$	NS $p = 1.000$	NE	NS $p = 0.373$	NS $p = 0.449$	S $p = 0.011$	PFS – NS $p = 0.350$ OS – NS $p = 0.058$	IHC

R – retrospective study, P – prospective study, R-P – retro-prospective study, O – observational study, C – cohort study, S – significant, NE – not examined, NS – not significant, OS – overall survival, PP – poor prognosis, DSS – disease-specific survival, DSD – disease-specific death, DFS – disease-free survival, PFS – progression-free survival, RFS – recurrence-free survival, IHC – immunohistochemistry, RT-qPCR – reverse transcription polymerase chain reaction.

with PD-L1 [17, 20, 30] and PD-L2 [33]. Only PD-L1 protein expression proved to be a prognostic factor. Overall survival [16, 24, 31, 35, 42], disease-free survival [16, 20, 45], progression-free survival [35], poor prognosis [26] and recurrence-free survival [42] were correlated with PD-L1 protein expression. Immunohistochemistry was the most commonly used diagnostic method.

Discussion

B7 protein family and the receptors

PD-L1 (B7-H1)/PD-L2 (B7-DC)/PD-1

B7-H1/PD-L1 protein (programmed cell death 1 ligand 1/cluster of differentiation 274/CD274/PDCD1LG1/B7H1/B7-H/PDCD1L1/PDCD1LG1/PDL1) is a type I membrane

Table 2. Results of MINORS for non-comparative studies

No.	Reference	MINORS for non-comparative studies								
		1	2	3	4	5	6	7	8	Score
1	Quan *et al.*, 2020 [15]	2	2	2	1	0	1	2	0	10
2	Ahmadi *et al.*, 2019 [18]	2	2	2	1	0	1	2	0	10
3	de Vincente *et al.*, 2019 [20]	1	2	2	0	0	0	2	0	7
4	Takahashi *et al.*, 2019 [22]	2	1	1	2	0	0	2	0	8
5	Tojyo *et al.*, 2019 [23]	2	1	2	2	0	1	2	0	10
6	Hanna *et al.*, 2018 [24]	2	1	2	2	0	1	2	0	10
7	Maruse *et al.*, 2018 [25]	2	1	2	2	0	1	2	0	10
8	Udeabor *et al.*, 2018 [28]	2	0	0	1	0	0	2	0	5
9	Wirsing *et al.*, 2018 [29]	2	2	1	2	0	0	2	1	10
10	Ahn *et al.*, 2017 [30]	2	1	2	2	0	2	2	0	11
11	Feng *et al.*, 2017 [31]	0	1	2	2	1	2	2	0	10
12	Foy *et al.*, 2017 [32]	2	2	2	2	0	1	2	0	11
13	Groeger *et al.*, 2017 [33]	2	1	2	1	0	0	2	0	8
14	Hirai *et al.*, 2017 [34]	2	1	0	0	0	0	2	0	5
15	Kogashiwa *et al.*, 2017 [35]	2	2	2	1	0	1	2	0	10
16	Kubota *et al.*, 2017 [36]	2	1	2	2	0	1	2	0	10
17	Mattox *et al.*, 2017 [37]	1	1	2	2	0	1	2	0	9
18	Takakura *et al.*, 2017 [38]	2	1	2	2	0	1	2	0	10
19	Troeltzsch *et al.*, 2017 [39]	2	2	2	0	0	1	2	0	9
20	Satgunaseelan *et al.*, 2016 [41]	2	2	2	2	0	1	2	0	11
21	Straub *et al.*, 2016 [42]	2	2	2	2	0	1	2	0	11
22	Chen *et al.*, 2015 [43]	2	2	2	2	0	1	2	0	11
23	Lin *et al.*, 2015 [44]	2	1	2	2	0	1	2	0	10
24	Oliveira-Costa *et al.*, 2015 [45]	2	2	2	2	0	2	2	0	12
25	Cho *et al.*, 2011 [46]	2	2	2	2	0	1	2	0	11

1 – a clearly stated aim, 2 – inclusion of consecutive patients, 3 – prospective data collection, 4 – endpoints appropriate to the aim of the study, 5 – unbiased assessment of the study endpoint, 6 – follow-up period appropriate to the aim of the study, 7 – loss to follow up less than 5%, 8 – prospective calculation of the study size; score: 0 – not reported, 1 – reported but inadequate, 2 – reported and adequate; the ideal global score for comparative studies is 16.

protein (mass 40 kDa) encoded by the *CD274* gene on chromosome 9 (locus 9p24.1). The B7-H1 protein has genes parallel to B7-1 ligand in 21%. PD-L1 has three domains: immunoglobulin constant-like domain (IgC; extracellular), the variable-like domain (IgV; extracellular) and homology domain for PD-1 [8, 49]. Intracellular structures are poorly studied. PD-L1 molecules are more prevalent than PD-L2. The PD-L1 protein suppresses the immune system [50–52]. PD-L1 protein is found on activated T lymphocytes, dendritic cells, B lymphocytes, NK cells, monocytes, macrophages, endothelial cells, epithelial cells, fibroblasts, mesenchymal stem cells, syncytiotrophoblasts, islets of Langerhans and neurons. The PD-L1 molecule plays a crucial role in the differentiation of regulatory T lymphocytes. The increase in its status is also associated with chronic inflammation and secretion of interferon γ (IFN-γ) [8, 33, 43, 53, 54]. It can affect the results of treatment of hepatitis B and C [55]. The presence of the PD-L1 protein expression has been demonstrated in glioma, ovarian cancer, renal cancer, head and neck cancer, breast cancer, sigmoid cancer, pancreatic cancer, non-small cell lung cancer and melanoma [53, 56]. Wang *et al.* demonstrated that PD-L1 positive expression was a prognostic factor for poor disease-specific survival in pancreatic carcinoma [53]. An increase in the response to anti-PD-1/PD-L1 therapies has been demonstrated in the treatment of lung cancer associated with smoking. An increased expression of PD-L1 refers to solid tumours, where it can serve as a defence of the tumour against the immune system [44]. In OSCC, PD-L1 protein expression was correlated with gender [16, 18, 35, 41, 44], grade [19, 48], stage [17, 21, 30], tumour size [21, 29, 30], nodal metastases [25, 26, 39–42], distant metastases [25, 26], localisation [41], vascular invasion [28], positive TILs infil-

Table 3. Results of MINORS for comparative studies

No.	Reference	MINORS for comparative studies												
		1	2	3	4	5	6	7	8	9	10	11	12	Score
1	Cui *et al.*,2020 [13]	2	1	0	1	0	1	2	0	0	0	0	2	9
2	Meehan *et al.*, 2020 [14]	2	0	0	1	0	2	2	0	0	1	1	2	11
3	Wilms *et al.*, 2020 [16]	2	0	2	1	0	2	2	0	0	1	2	2	14
4	Zhao *et al.*, 2020 [17]	1	2	2	0	0	1	2	0	1	1	2	2	14
5	Chen *et al.*, 2019 [19]	2	1	2	2	0	1	2	0	2	0	1	2	15
6	Kouketsu *et al.*, 2019 [21]	2	1	2	0	0	0	2	0	2	1	1	2	13
7	Weber *et al.*, 2019 [48]	2	1	2	2	2	2	2	0	2	0	1	2	18
8	Stasikowska-Kanicka *et al.*, 2018 [26]	2	2	2	0	0	1	2	0	1	1	1	2	14
9	Stasikowska-Kanicka *et al.*, 2018 [27]	2	2	2	0	0	1	2	0	1	1	1	2	14
10	Weber *et al.*, 2017 [40]	2	1	2	0	0	1	2	0	1	1	1	2	13
11	Malaspina *et al.*, 2011 [47]	1	2	2	0	0	1	2	0	1	1	1	2	13

1 – a clearly stated aim, 2 – inclusion of consecutive patients, 3 – prospective data collection, 4 – endpoints appropriate to the aim of the study, 5 – unbiased assessment of the study endpoint, 6 – follow-up period appropriate to the aim of the study, 7 – loss to follow up less than 5%, 8 – prospective calculation of the study size, 9 – an adequate control group, 10 – contemporary groups, 11 – baseline equivalence of groups, 12 – adequate statistical analyses; score: 0 – not reported, 1 – reported but inadequate, 2 – reported and adequate; the ideal global score for comparative studies is 24.

tration [39], recurrence [42], disease-specific survival [45], disease-free survival [16, 20, 45], recurrence-free survival [42], overall survival [16, 24, 31, 35, 42], progression-free survival [24] and poor survival [26]. Lin *et al.* suggested the possibility of using PD-L1 as a prognostic factor especially in smokers and men [44]. No correlation was found between the PD-L1 protein expression and betel chewing, alcohol consumption, perineural invasion, depth of invasion, treatment, or distant metastases [41, 44]. A high expression is associated with better overall survival [30]. In addition, the presence of PD-L1 and TILs expression has been correlated with better outcome in patients with locally advanced OSCC. In those cases, the risk of recurrence was lower and survival was improved [24, 35].

B7-DC/PD-L2 protein (programmed cell death 1 ligand 2/cluster of differentiation 273/CD273/PDCD1LG2/B7DC/Btdc/PDCD1L2/PDL2/bA574F11.2) is encoded by the *PDCD1LG2* gene on chromosome 9 (locus 9p24.1). The B7-DC protein has genes parallel to B7-1 ligand in 23%. PD-L2 has three domains: immunoglobulin constant-like domain (IgC; extracellular), the variable-like domain (IgV; extracellular) and homology domain for PD-1 [8, 57]. Intracellular structures are poorly studied [50–52]. PD-L2 protein is found on dendritic cells, B lymphocytes, Th2 cells, monocytes, macrophages, mast cells, hepatocytes and endothelial cells. This protein suppresses the immune system by inhibiting the T cell response through PD-1 binding. The presence of this molecule on tumour cells may cause the tumour resistance to the immune system [7, 8, 58]. PD-L2 expression was correlated with stage, but not associated with tumour size, nodal metastases, grade, progression-free survival or overall survival in OSCC [33, 35, 48].

Programmed cell death protein 1 receptor is a transmembrane protein (PD-1/cluster of differentiation 279/CD279; 50-55 kDa) encoded by the *PDCD1* gene on chromosome 2 (locus 2q37.3). It consists of five domains including ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITSM (immunoreceptor tyrosine-based switch motif). The IgV domain has genes parallel to CTLA-4 receptor in 21-33% and to CD28 receptor in 15.6% [8, 59]. The PD-1 receptor is found in activated T cells, B lymphocytes, NK cells, mast cells, macrophages and dendritic cells [60]. PD-1 proteins were not detected in immature T lymphocytes. The presence of PD-1 protein on TILs was revealed more frequently in comparison with peripheral blood. This was regardless of the patient's age or HPV infection [61]. PD-1 generates an inhibitory signal that regulates the functions of T lymphocytes. This receptor has two known ligands: PD-L1 and PD-L2 [58]. Programmed cell death protein 1 receptor does not directly affect apoptosis or cell survival. PD-1 signals regulate the cellular response, but this is not completely clear. The signalling process is different in B and T lymphocytes. After binding by the PD-1 receptor, the ligand is followed by phosphorylation of tyrosine in the ITSM domain and recruitment of the SHP-2 (Src homology region 2 domain-containing phosphatase-1) and SHP-1 signal molecules (Src homology region 2 domain-containing phosphatase-2). This blocks the activation of PI-3K molecules (phosphatidylinositol 3-kinase) and ZAP70 (zeta chain-associated protein kinase 70) [58]. Activation of SHP-2 causes dephosphorylation of the molecule involved in TCR receptor activity and as a result reduces signal and cytokine synthesis [58]. Higher prevalence of PD-1 receptors on

activated lymphocytes suggests that it is more important than the CTLA-4 receptor pathway [62]. An inflammatory reaction induces an increase in the expression of PD-L1 and PD-L2 proteins. They can serve as a feedback mechanism to reduce T cell responses in tissues and protect them from auto-aggressive damage. The binding of PD-L1 ligand to PD-1 receptor leads to the inhibition of proliferation and the reduction of IFN-γ and IL-10 cytokine secretion (interleukin 10) by about 80%, and IL-2 (interleukin 2) to the threshold below the reference values. This inhibits the proliferation of lymphocytes and promotes the survival of the cancer cell [63]. The level of PD-1 protein is high on activated lymphocytes, but it quickly decreases after removal of the antigen. However, when lymphocytes have to contend with chronic inflammation (infection or cancer), the expression of PD-1 protein is still high, which causes "exhaustion" of lymphocytes. A high oestrogen level can also induce PD-1 receptors on T cells and APC [64]. The inhibitory effect was found on Th (CD4+; helper cells) and Tc (CD8+; cytotoxic cells) lymphocytes [65]. The presence of the PD-1 protein expression has been demonstrated in bladder cancer [65] and pregnancy-associated melanoma [66]. In OSCC, PD-1 protein expression was correlated with age [30], stage [30], nodal metastases [30], perineural invasion [30] and not related to the disease-free survival [30], recurrence-free survival [42] or overall survival [30, 42]. PD-1 signalling was strongly enriched in never-smokers and never-drinkers [30]. The role of the PD-1/PD-L1 pathway is not limited to the pathogenesis of tumours. This pathway is important in various diseases: insulin-dependent diabetes mellitus, lupus erythematosus, myocarditis, inflammation of the brain and spinal cord, rheumatoid arthritis and inflammatory bowel diseases [67].

B7 pathway inhibition in cancers and OSCC

The B7/CD28 pathway influences the regulation of the immune response by limiting the time and strength of the inflammatory response. Although the co-stimulation mechanism of the B7-CD28 pathway is not known, monoclonal antibodies are currently used in targeted therapies of malignant tumours, autoimmune and infectious diseases. In cancer, CTLA-4 and PD-1 receptors are blocked by ligands (B7-1 and B7-2 for CTLA-4, and PD-L1 and PD-L2 for PD-1). As a result of ligand-receptor binding, neoplastic lesions are not recognized by the immune system. The function of monoclonal antibodies is to block CTLA-4 and PD-1 receptors. As a result, T lymphocytes re-recognize tumour antigens. The immunomodulatory drugs that block the CTLA-4 protein are ipilimumab and tremelimumab, while the PD-1 is blocked by nivolumab, pembrolizumab, pidilizumab, BMS-936559, MEDI4736 (durvalumab) and MPDL3280A [49, 56, 68–72]. They are mainly used in the treatment of melanoma [66, 70], lung cancer [66, 70], genitourinary cancer [66] and prostate cancer [72]. Targeted immunotherapy in head and neck melanoma improved survival. Ipilimumab and nivolumab had a better effect together than in monotherapy [56]. Nivolumab was used in a clinical trial of 296 melanoma, non-small-cell lung cancer, prostate cancer, renal cancer, and colorectal cancer patients. The positive tumour response (expression of PD-L1 in > 5% of cells) was seen in 18% of patients with non-small cell lung cancer, 28% with melanoma and in 27% with renal-cell carcinoma [56]. In Carbognin *et al.* study, nivolumab, pembrolizumab and MPDL3280A were studied in patients with melanoma, lung and genitourinary cancers. The overall response rate was significantly higher in patients with positive PD-L1 expression [68].

The role of immunomodulatory drugs in the treatment of oral cancer remains unclear and requires more research. The oral squamous cell carcinoma is highly immunosuppressive. An anti-PD-1 monoclonal antibody therapy may result in better clinical efficacy in OSCC patients [73–75]. Foy *et al.* studied the clinical response to pembrolizumab in HPV-negative oral squamous cell carcinoma. The PD-L1 protein was overexpressed and the score of response to pembrolizumab was higher in never-smokers and never-drinkers than in smokers and drinkers, although the mutational load was lower in never-smokers and drinkers. The main difference between oral squamous cell carcinoma in never-smokers and never-drinkers when compared to smokers and drinkers, lies in the immune microenvironment, suggesting a higher clinical benefit of PD-L1 inhibition in oral cancer in never-smokers and drinkers. The immune checkpoint inhibitors can probably extend the survival of many patients [32].

Conclusions

The biology of squamous cell carcinoma is unknown. The search for new molecular markers is extremely important. Components of the B7 family are potential objects of research. Any mutations in gene encoding PD-L1 and quantitative changes in the status of PD-L1 protein may have an impact on the prognosis of oral squamous cell carcinoma.

Conflict of interest

The authors declare no conflict of interest.

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Original paper

The B7 family molecules in oral squamous cell carcinoma: a systematic review. Part II: B7-1, B7-2, B7-H2, B7-H3, B7-H4, B7-H5 (VISTA), B7-H6 and B7-H7

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Abstract

Introduction: The most common malignant neoplasm of the oral cavity is squamous cell carcinoma. It accounts for over 95% of malignant lesions in this area and is characterised by rapid spread and distant metastases.
Aim: To collect and review the data on B7 family proteins as prognostic factors in oral squamous cell carcinoma and to describe their role in aggressive disease progression.
Material and methods: A systematic review of the literature about B7-1, B7-2, B7-H2, B7-H3, B7-H4, B7-H5 (VISTA), B7-H6 and B7-H7, was carried out, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. 9 articles published before 22 May 2020 were included in the systematic review.
Results: The biggest study group consisted of 201 patients and the smallest – 40 patients. B7-1, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7 protein expressions were prognostic factors. Overall survival was significantly associated with B7-H3, B7-H5, B7-H6 and B7-H7. Immunohistochemistry was the diagnostic method used in all the studies.
Conclusions: The data presented indicate the complexity of cellular and molecular processes related to the formation, development and invasion of oral cancer. Mutations in genes encoding the B7 family and changes in the B7 protein pathway may affect the prognosis of survival in patients with oral squamous cell carcinoma.

Key words: squamous cell carcinoma, oral cavity, B7 antigens.

Introduction

The most common malignant neoplasm of the oral cavity is squamous cell carcinoma (oral squamous cell carcinoma – OSCC), which accounts for over 95% of these lesions. The lesions are more common in men over 60 years of age. Oral cancer is characterised by rapid spread and distant metastases. The outcome is still unsatisfactory. Identification of new markers may be of use in prevention, diagnostics, prognosis and choice of a targeted therapy [1–4].

The B7/CD28 family proteins are involved in the regulation of the immune response by limiting both time and strength of the inflammatory response [5, 6]. The B7 family includes proteins: B7-1, B7-2, B7-H2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7. The group of receptors includes proteins: CD28, CTLA-4, ICOS, PD-1 and TMIGD2 (Table 1). They are potential objects of research when introducing new therapeutic agents [7–9].

Aim

The aim of this paper was to systematically review the literature and to summarise correlations between B7 family proteins and prognosis in OSCC.

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Table 1. B7/CD28 family

Ligands (B7)	Receptors (CD28)
B7-1 (cluster of differentiation 80/CD80/B7/B7.1/BB1/CD28LG/CD28LG1/LAB7)	CD28 (cluster of differentiation 28) CTLA-4 (cytotoxic T-lymphocyte-associated protein 4/cluster of differentiation 152/CD152) PD-L1 (programmed cell death 1 ligand 1/cluster of differentiation 274/B7-H1/CD274/PDCD1LG1/B7H1/B7-H/PDCD1L1/PDCD1LG1/PDL1)
B7-2 (cluster of differentiation 86/CD86/B7.2/B70/CD28LG2/LAB72)	CD28 (cluster of differentiation 28) CTLA-4 (cytotoxic T-lymphocyte-associated protein 4/cluster of differentiation 152/CD152)
B7-DC (programmed cell death 1 ligand 2/cluster of differentiation 273/CD273/PDCD1LG2/B7DC/Btdc/PDCD1L2/PDL2/bA574F11.2)	PD-1 (cluster of differentiation 279/CD279) RGMb (repulsive guidance molecule B)
B7-H1 (programmed cell death 1 ligand 1/cluster of differentiation 274/PD-L1/CD274/PDCD1LG1/B7H1/B7-H/PDCD1L1/PDCD1LG1/PDL1)	PD-1 (cluster of differentiation 279/CD279) B7-1 (cluster of differentiation 80/CD80/B7/B7.1/BB1/CD28LG/CD28LG1/LAB7)
B7-H2 (cluster of differentiation 275/CD275/ICOSLG/B7H2/B7RP-1/B7RP1/GL50/ICOS-L/ICOSL/LICOS/inducible T cell co-stimulator ligand)	ICOS (inducible T cell co-stimulator/cluster of differentiation 278/CD278)
B7-H3 (cluster of differentiation 276/CD276/4Ig-B7-H3/B7H3/B7RP-2)	Unknown
B7-H4 (B7x/B7s/VTCN1/B7H4/B7S1/B7X/B7h.5/PRO1291/VCTN1/V-set domain containing T cell activation inhibitor 1)	Unknown
B7-H5 (V-domain Ig suppressor of T cell activation/VISTA/VSIR/B7H5/GI24/PP2135/SISP1/DD1alpha/C10orf54/chromosome 10 open reading frame 54/PD-1H/V-set immunoregulatory receptor)	Unknown
B7-H6 (NCR3LG1)	NKp30 (natural cytotoxicity triggering receptor 3/NCTR3/CD337/cluster of differentiation 337)
B7-H7 (BTNL2/BTL-II/HHLA2/B7H7/B7-H5/B7y)	CD28H (transmembrane and immunoglobulin domain containing 2/TMIGD2/IGPR-1)

Material and methods

For this review, a systematic search of the literature was conducted in the PubMed, Web of Science, Scopus, Embase, Cochrane Library, and Google Scholar databases to identify papers containing data about B7 family proteins in OSCC. The PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines were used [10].

PICO for study characteristics was used (P – patient/population/problem; I – intervention/exposure; C – comparison; O – outcome):

P: at least 10 patients with primary OSCC;

I: protein expression evaluation;

C: not required;

O: B7 family proteins as prognostic factors in OSCC – only B7-1, B7-2, B7-H2, B7-H3, B7-H4, B7-H5 (VISTA), B7-H6 and B7-H7.

Inclusion criteria for papers were as follows: at least 10 patients with primary OSCC, B7 protein expression evaluation, study conducted only on humans, in the English language, prospective and retrospective studies, clinical studies and immunohistochemical evaluation of B7 protein. Articles were excluded if specimens derived from OSCC recurrences (not primary tumours), research was conducted on cell lines, was not conducted on humans, the study group consisted of less than 10 patients, non-B7 family protein expression was evaluated or the study was not in English. Duplicate records as well as letters and papers that did not contain significant information were also excluded.

A retrospective analysis of articles on the B7 family proteins as risk factors in OSCC published from 2012 to 22 May 2020 was performed. Key words: "B7 family", "CTLA-4/B7-1/B7-2 pathway", "B7-H2/CD275/ICOSLG/B7H2/B7RP-1/B7RP1/GL50/ICOS-L/ICOSL/LICOS/inducible T cell co-stimulator ligand", "B7-H3/CD276/4Ig-B7-H3/B7H3/B7RP-2", "B7-H4/B7x/B7s/VTCN1/B7H4/B7S1/B7X/B7h.5/PRO1291/VCTN1/V-set domain containing T cell activation inhibitor 1", "B7-H5/V-domain Ig suppressor of T cell activation/VISTA/VSIR/B7H5/GI24/PP2135/SISP1/DD1alpha/C10orf54/chromosome 10 open reading frame 54/PD-1H/V-set immunoregulatory receptor", "B7-H6/NCR3LG1", "B7-H7/BTNL2/BTL-II/HHLA2/B7H7/B7-H5/B7y" and oral "cancer/OSCC/oral squamous cell carcinoma" were used. Articles were screened and sorted based on titles and abstracts. Then, articles were evaluat-

ed for eligibility. Data extracted from those records were analysed in detail. The following pieces of information were collected: total patient number, occurrence of B7 family alterations in OSCC, correlations with age, gender, grading, primary tumour size (T stage), nodal metastases (N stage), staging, prognostic significance and diagnostic methods (immunohistochemistry and other methods). For randomized studies, the Cochrane Collaboration tool was used [11] and the methodological index for non-randomized studies (MINORS) was used [12]. The ideal global score for non-comparative studies is 16 and for comparative is 24 [12].

Statistical analysis

Statistical analysis was carried out using the Statistica 13.3 (StatSoft Inc. Tulsa, United States), licensed by the the Medical University of Gdansk.

Results

In the first step of selection 261 references were identified. 248 records were selected after the exclusion of the duplicates. Eventually, 9 articles were included in the systematic review (Figure 1) [13–21]. Three studies addressed B7-1 protein, one article was on B7-2, 2 studies involved B7-H3, one article was on B7-H4, one study involved B7-H5, one study involved B7-H6 and one involved B7-H7 (some articles were duplicated). Currently, B7-H2 protein is not described in OSCC. The first identified study was published in 2012. Table 2 shows the articles included in the analysis [13–21].

Observational studies were the most commonly presented papers. There were no randomized controlled trials. 88.89% were retrospective (n = 8) and 11.11% of the studies were prospective (n = 1). Studies were mostly cohort-based (88.99%, n = 8), rarely cross-sectional (2.23%, n = 1) (Tables 3 and 4).

The biggest study group consisted of 201 patients and the smallest – 40 patients. In total, 893 patients (excluding duplicates) were analysed in the studies. The following proteins have been described: B7-1, B7-2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7 (Table 2). In all tested samples, the expression of proteins was found by testing proteins B7-H4 and B7-H5. The occurrence of protein expression was as follows: B7-1 – 55%; B7-2 – 57.5%; B7-H3 – about 67%, B7-H4 – 100%; B7-H5 – 100%, B7-H6 – 48% and B7-H7 – no data. Only B7-1 expression was correlated with age [13]. Grading was correlated with B7-H3 [17], B7-H4 [18] and B7-H5 [19] protein expression. Primary tumour size (T stage) was associated with B7-H3 protein [16]. Metastases in lymph nodes were correlated with B7-1 [14], B7-H3 [17], B7-H4 [18] and B7-H5 protein expression [19]. Staging was correlated with B7-1, B7-2 [14] and B7-H3 protein expression [17]. B7-1, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7 protein expressions were prognostic factors. Overall survival was significantly

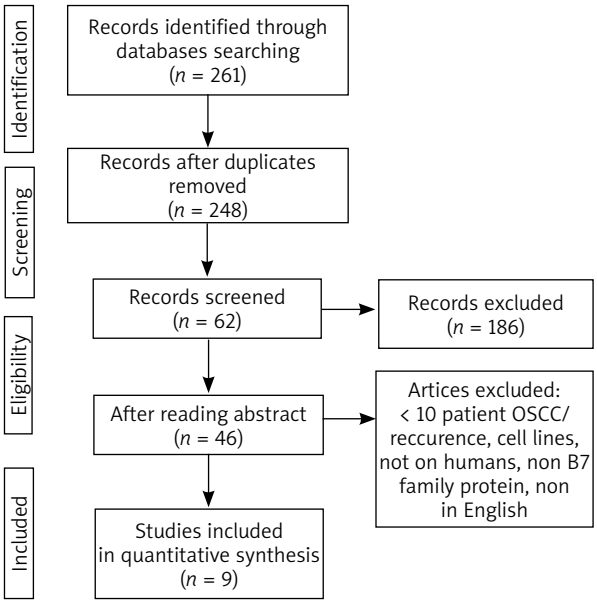

Figure 1. PRISMA flow diagram of study selection

associated with B7-H3 [17], B7-H5 [19], B7-H6 [20] and B7-H7 [21]. Disease-free survival was correlated with B7-H6 protein [56]. Poor survival was related to B7-H4 [18]. Relapse-free survival was significantly associated with B7-1 [13]. Immunohistochemistry was used as a diagnostic method in all the studies.

Discussion

B7 protein family and the receptors

B7-1/B7-2/CTLA-4 pathway

B7-1 protein is a type I membrane protein encoded by the *CD80* gene on chromosome 3 (locus 3q13.33). B7-1 has two domains: immunoglobulin constant-like domain (IgC; extracellular) and the variable-like domain (IgV), required for receptor binding. This protein is expressed on the surface of monocytes, macrophages, mast cells, activated B lymphocytes, podocytes, fibroblasts, antigen-presenting and dendritic cells. T cells are co-stimulated and activated by B7-1 protein. The function of the CD80 protein is to stimulate the signal that activates T lymphocytes. This is necessary for their survival. B7-1 protein binds to CD28, CTLA-4 and PD-L1 receptors. The binding of the CD80 protein to the CD28 receptor enables autoregulation and intercellular connection. The binding to CTLA-4 receptor allows reduction of regulation and cellular dissociation. B7.1 protein plays a role in the induction of T cell anti-tumour immunity [8, 9, 13, 15, 22]. The presence of the B7-1 protein expression has been demonstrated in numerous malignancies, such as human hepatocellular cancer [22], thyroid cancer [23], bladder cancer [24] and pancreatic cancer [25]. Chaux *et al.* revealed that

Table 2. B7 family genetic alterations in oral squamous cell carcinomas: a review of current studies

No.	Reference	Study type	Total patient number	Occurrence of protein expression *n* patients (%)	Correlations							Methods
					Age	Gender	Grading	Primary tumour size (T stage)	Nodal metastases (N stage)	Staging	Prognostic significance	
B7-1:												
1	Rah *et al.*, 2018 [13]	O, R, C	60	No data	S $p = 0.016$	NS $p = 0.521$	NE	NS $p = 0.889$	NS $p = 0.233$	NS $p = 0.655$	ReFS – S $p = 0.038$ DFS – NS $p = 0.156$	IHC
2	Zhang *et al.*, 2017 [14]	O, P, C	40	22 (55%)	NE	NE	NS $p > 0.050$	NE	S $p = 0.036$	S $p = 0.020$	NE	IHC
3	Dayan *et al.*, 2012 [15]	O, R, C	64	No data	NE	NE	NE	NE	NE	NE	NE	IHC
B7-2:												
1	Zhang *et al.*, 2017 [14]	O, P, C	40	23 (57.5%)	NE	NE	NS $p > 0.050$	NE	S $p = 0.024$	S $p = 0.041$	NE	IHC
B7-H2:												
NE												
B7-H3:												
1	Chen *et al.*, 2015 [16]	O, R, C	72	48 (67%)	NS $p > 0.050$	NS $p > 0.050$	NE	S $p = 0.0001$	NS $p > 0.050$	S $p = 0.004$	OS – S $p = 0.005$	IHC
2	Zhang *et al.*, 2015 [17]	R, CSS	76	51 (67.1%)	NE	NE	S $p = 0.011$	NS $p = 0.135$	S $p = 0.026$	NS $p = 0.135$	NE	IHC
B7-H4:												
1	Wu *et al.*, 2016 [18]	O, R, C	165 primary OSCC 12 recurrent OSCC 10 OSCC with pre-surgical radiotherapy 17 OSCC with pre-surgical chemotherapy	165 (100%)	NE	NE	S $p < 0.010$	NS $p = 0.0683$	S $p < 0.050$	NS $p = 0.068$	PS – S $p < 0.050$	IHC
B7-H5 (VISTA):												
1	Wu *et al.*, 2017 [19]	O, R, C	165 primary OSCC 12 recurrent OSC 10 OSCC with pre-surgical radiotherapy 17 OSCC with pre-surgical chemotherapy	165 (100%)	NE	NE	NS $p > 0.050$	NS $p > 0.050$	S $p < 0.050$	NS $p > 0.050$	OS – S $p = 0.036$	IHC
B7-H6:												
1	Wang *et al.*, 2017 [20]	O, R, C	50	24 (48%)	NS $p > 0.050$	NS $p > 0.050$	S $p = 0.0186$	NS $p > 0.050$	NS $p > 0.05$	NS $p = 0.460$	DFS – S $p = 0.005$ OS – S $p = 0.007$	IHC
B7-H7:												
1	Xiao *et al.*, 2019 [21]	O, R, C	201	No data	NE	NE	NS	NS	NS	NE	OS – S $p = 0.031$	IHC

R – retrospective study, P – prospective study, O – observational study, C – cohort study, CSS – cross-sectional study, S – significant, NE – not examined, NS – not significant, OS – overall survival, PS – poor survival, PP – poor prognosis, DSS – disease-specific survival, DSD – disease-specific death, DFS – disease-free survival, PFS – progression-free survival, RFS – recurrence-free survival, ReFS – relapse-free survival, IHC – immunohistochemistry, RT qPCR – reverse transcription polymerase chain reaction.

Table 3. Results of MINORS for non-comparative studies

No.	Reference	MINORS for non-comparative studies								
		1	2	3	4	5	6	7	8	Score
1	Zhang *et al.*, 2017 [14]	2	2	2	1	0	1	2	0	10
2	Dayan *et al.*, 2012 [15]	2	2	2	2	0	1	2	0	11

1 – a clearly stated aim, 2 – inclusion of consecutive patients, 3 – prospective data collection, 4 – endpoints appropriate to the aim of the study, 5 – unbiased assessment of the study endpoint, 6 – follow-up period appropriate to the aim of the study, 7 – loss to follow up less than 5%, 8 – prospective calculation of the study size; score: 0 – not reported, 1 – reported but inadequate, 2 – reported and adequate; the ideal global score for comparative studies is 16.

Table 4. Results of MINORS for comparative studies

No.	Reference	MINORS for comparative studies												
		1	2	3	4	5	6	7	8	9	10	11	12	Score
1	Xiao *et al.*, 2019 [21]	2	1	2	2	1	1	2	0	1	1	1	2	16
2	Rah *et al.*, 2018 [13]	2	2	2	2	2	1	2	0	2	2	2	2	21
3	Wang *et al.*, 2017 [20]	2	1	2	2	0	1	2	0	1	1	1	2	15
4	Wu *et al.*, 2017 [19]	2	2	2	2	0	1	2	0	1	1	1	2	16
5	Wu *et al.*, 2016 [18]	2	2	2	2	0	1	2	0	1	1	1	2	16
6	Chen *et al.*, 2015 [16]	2	0	2	2	0	1	2	0	1	1	1	2	14
7	Zhang *et al.*, 2015 [17]	2	2	2	0	0	1	2	0	2	2	2	2	17

1 – a clearly stated aim, 2 – inclusion of consecutive patients, 3 – prospective data collection, 4 – endpoints appropriate to the aim of the study, 5 – unbiased assessment of the study endpoint, 6 – follow-up period appropriate to the aim of the study, 7 – loss to follow up less than 5%, 8 – prospective calculation of the study size, 9 – an adequate control group, 10 – contemporary groups, 11 – baseline equivalence of groups, 12 – adequate statistical analyses; score: 0 – not reported, 1 – reported but inadequate, 2 – reported and adequate; the ideal global score for comparative studies is 24.

in colorectal cancer, expression of the B7-1 and B7-2 molecules were not found. As a consequence, the immune system does not recognise cancer cells as antigenic [26, 27]. Wang *et al.* demonstrated that the B7-1 negative status was a prognostic factor for poor disease-specific survival in pancreatic carcinoma [25]. The literature documenting B7-1 protein in OSCC is limited. CD80 protein in OSCC was first reported in 2012 by Dayan *et al.* [15]. According to some studies, the percentage of common B7-1 protein range was 55% [14]. B7-1 protein expression correlated with age, recurrence, relapse-free survival [13], stage, and nodal metastases in OSCC [14].

B7-2 is a type I membrane protein encoded by the *CD86* gene on chromosome 3 (locus 3q13.33). This protein is expressed on the surface of antigen-presenting cells, T cells, B lymphocytes, dendritic cells, monocytes, macrophages and mast cells. It is the source of the co-stimulatory signal. The B7-2 protein has genes parallel to B7-1 ligand in 27%. It activates T lymphocytes and is necessary for their survival. B7-2 protein binds to CD28 and CTLA-4 receptors. The binding of the CD86 protein to the CD28 receptor enables autoregulation and intercellular connection. The binding to CTLA receptor allows to reduce regulation and cellular dissociation. B7.1 protein plays a role in the induction of T cell anti-tumour immunity [7, 14, 22]. The presence of the B7-2 protein expression has been demonstrated in human hepatocellular carcinoma [27] and thyroid carcinoma [23]. B7-2 protein in OSCC was reported in 2017 by Zhang *et al.* In the study mentioned, the percentage of common B7-2 protein was 57.5%. This protein expression correlated with stage and nodal metastases [14].

CD28 is a co-stimulatory receptor of B7-1 and B7-2 molecules. CD28 is encoded by the *CD28* gene on chromosome 2 (locus 2q33.2). This protein consists of the immunoglobulin variable-like domain (IgV; extracellular), transmembrane domain, stalk domain and a cytoplasmic domain. CD28 receptor is found on naive T lymphocytes, plasma cells, NK and NKT cells (natural killers T cells). This molecule is a receptor of B7-1 and B7-2 ligands. This protein stimulates T cell receptor (TCR). CD28 molecule is associated with T cell activation, proliferation, tolerance and survival. This protein provides a signal for production of various interleukins (e.g. interleukin 2) and amplifies the immune system response. Activated T cells stimulate B lymphocytes to produce antibodies. The pathways associated with CTLA-4 and ICOS cannot be activated without the induction of the CD28 receptor [8, 28–30]. CD28 protein affects apoptosis *via* Bcl-xL (anti-apoptotic protein – B-cell lymphoma-extra large). Lymphocytes are not subject to programmed death [31]. Activation of CD28 causes the induction of transcription and/or stabilization of cytokine mRNAs: IL-4, IL-5, IL-8, IL-13, interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α). The lack of co-stimulation by CD28 leads to the anergy of clonal lymphocytes. Anergy means a lack of reaction by the body's defence mechanisms to foreign substances [29, 30].

CTLA-4 is a protein receptor of B7-1 and B7-2 molecules and down-regulates T lymphocytes activity. CTLA-4 is encoded by the *CTLA-4* gene located on chromosome

2 in locus 2q33.2. The CTLA-4 has genes parallel to the CD28 receptor in 26.9% [8, 32, 33]. This protein consists of the immunoglobulin variable-like domain (IgV; extracellular), transmembrane domain, stalk domain and a cytoplasmic domain. CTLA-4 receptor is found on T lymphocytes, dendritic cells, B lymphocytes, NK cells, NKT cells, monocytes, granulocytes and fibroblasts. Roles of CTLA-4 include inhibition of T cell activation and their response by blocking AKT protein (serine-threonine protein kinase = protein kinase B). CTLA protein skips phosphatidyl-inositol 3-kinase signalling pathway. Most CTLA-4 molecules are found in lysosomes. First, binding of B7-1 and B7-2 ligands to the CD28 receptor occurs. It activates the lymphocyte. Then, the CTLA-4 receptor is transported to the cell membrane and expressed. Activation of CTLA-4 provides a stimulus that can trigger feedback to inhibit lymphocyte activation. The presence of the CTLA-4 receptor on the tumour cell membrane avoids a specific immune response [8]. Excessive expression of the gene encoding the CTLA-4 protein may be associated with: Ewing's sarcoma, laryngeal, hepatocellular, prostate, lung, breast, cervical [6, 32, 34–37], nasopharyngeal [38], and head and neck carcinoma [39]. In OSCC studies, the *CTLA-4* gene polymorphism was detected *via* polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Single nucleotide polymorphisms (SNPs): -1722C/T, -1661A/G, -1661G/G, -1661A/A and -318C/T in the promoter region, 49A/G in exon 1, and CT60A/G in the 3'untranslated region (UTR) were assessed in patients with tobacco-related carcinoma. Genotypes: -1722CC, -1661AG and CT60AA are more frequent in patients with OSCC. The polymorphism of the CTLA-4 1661-genotype alone and in combination with other polymorphisms may be a risk factor for oral cancer [30, 32]. In Moreira *et al.* study, no statistical association was found between overall survival and expression level of CTLA-4 protein [40].

B7-H2 and ICOS

B7-H2 is encoded by the *ICOSLG* gene on chromosome 21 (locus 21p12). The B7-H2 protein has genes parallel to B7-1 ligand in 27%. It binds to ICOS. B7-H2 was found on B lymphocytes, T cells, monocytes, mast cells, dendritic cells, macrophages, endothelial cells, epithelial cells, fibroblasts and osteoblasts. It plays an important role in cell-to-cell signalling, immune response, cell proliferation, functions of effector and Treg lymphocytes. CD275 can promote T lymphocyte production of cytokines, including IL-10, IL-4, IL-5, IL-7, IL-10, and interferon-γ. High levels of B7-H2 protein have been demonstrated in patients with colon cancer [8, 41], but have not been examined in OSCC.

ICOS is a co-stimulatory molecule and receptor of B7-H2 protein. This molecule is encoded by the *ICOS* gene on chromosome 2 (locus 2q33.2). The ICOS has genes parallel to CD28 receptor in 24.1% [8, 29, 30]. This protein consists of the immunoglobulin variable-like domain (IgV; extracellular), transmembrane domain, stalk domain and a cytoplasmic domain [8]. ICOS protein is expressed on activated T and B lymphocytes and binds to B7-H2 protein. It plays an important role in cell-to-cell signalling, immune response and cell proliferation. ICOS is upregulated on activated T lymphocytes. The gene polymorphism of *ICOS* was studied in positions +637 A/C and +1599 C/T [8, 29, 30].

B7-H3

B7-H3 protein is a type I membrane protein encoded by the *CD276* gene on chromosome 15 (locus 15q24.1). The B7-H3 protein has genes parallel to B7-1 ligand in 29%. It has two domains: IgV and IgC. B7-H3 molecule connects to the previously unknown receptor [7, 8, 42]. TLT-2 (TREML2) may be a binding partner for B7-H3. It may affect the growth of CD4 (T helper lymphocytes) and CD8 (T cytotoxic lymphocytes) T cells proliferation, and selectively increases the secretion of IFF. B7-H3 protein is found on activated dendritic cells, monocytes, macrophages, T, B and NK lymphocytes, epithelial cells, fibroblasts and osteoblasts. B7-H3 molecule is a protein that controls the immune system. There are studies suggesting stimulating effects (by T lymphocytes) as well as inhibiting the immune system (by NK cells). The B7-H3 protein induces selective production of interferon-γ. Inhibition of the immune system can occur through NFAT (nuclear factor of activated T cells), NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells), and AP-1 (activator protein 1) factors. This affects the TCR, which regulates gene transcription [7, 8, 43]. The presence of the B7-H3 protein expression has been demonstrated in neuroblastoma, lung cancer [44], pancreatic cancer [45], breast cancer [46], ovarian cancer [47], endometrial cancer [48], prostate cancer [49], renal cancer [50], gastric cancer [51], colorectal cancer [52], gallbladder cancer [53], hepatocellular cancer [54], and head and neck cancer [55]. It results in poor outcome in patients with these types of cancer [7]. Potential anti-B7-H3 therapy seems to be beneficial for the treatment of central nervous system tumours. The expression of B7-H3 can be associated with a decrease in the number of active lymphocytes, a large tumour size and the presence of metastases [56]. B7-H3 protein level in OSCC was higher than in normal mucosa [17]. In OSCC, B7-H3 protein expression was associated with grade [17], tumour size [16], nodal metastases [17], and overall survival [16]. B7-H3 was correlated with poor overall survival [16]. Protein expression was not associated with location of cancer, distant metastases, recurrence, alcohol consumption, betel chewing or smoking [16].

B7-H4

B7-H4 protein is a type I membrane protein encoded by the *B7-H4* gene on chromosome 1 (locus 1q13.1). The B7-H4 protein has genes parallel to B7-1 ligand in

21%. B7-H4 has two domains: IgC and IgV. Like B7-H3, B7-H4 is present in most tissues and is a control protein of the immune system. This protein was found on dendritic cells, monocytes, macrophages and mesenchymal stem cells. However, the protein expression itself is very limited [7, 8, 57]. B7-H4 inhibits the proliferation of CD4 T cells, resulting in suppressed production of cytokines (IL-2), and the proliferation of CD8 upon TCR/CD28 ligation. It binds to the receptor only on activated T cells, which can be a factor differentiating from other B7/CD28 family proteins. This suggests that the B7-H4 pathway may be another system that inhibits the cellular response of T lymphocytes. B7-H4 expression is correlated with an increased expression of IL-6 [7, 58, 59]. The presence of the B7-H4 protein expression has been demonstrated in lung cancer [60], ovarian cancer [61], cervical cancer [62], oesophagus cancer [63], gastric cancer [64], colorectal cancer [65], pancreatic cancer [66], prostate cancer [49], thyroid cancer [67] and melanoma [68]. B7-H4 protein level in OSCC was higher than in normal mucosa. B7-H4 protein expression was associated with grade and nodal metastases [18]. B7-H4 expression was an independent prognostic factor and associated with poor overall survival [18].

B7-H5 (VISTA)

B7-H5 protein is a type I membrane protein (50 kDa) encoded by the *C10orf54* gene on chromosome 10 (locus 10q22.1). The B7-H5 protein has genes parallel to B7-1 ligand in 24%. VISTA has two domains: IgC and IgV. VISTA transcription is regulated by the p53 protein. It is a control protein of the immune system. There are reports that the VISTA may function as a ligand and as a receptor [7, 8, 19, 69–72]. VISTA was found in dendritic cells, T lymphocytes, monocytes, macrophages, bone marrow cells and granulocytes. It is not present on B or NK cells. VISTA has inhibitory effects on T cell activity, proliferation, cytokine production, but not on B lymphocytes. Increased levels of VISTA proteins were observed in T lymphocytes in autoimmune diseases, cancer and generalized inflammation [8, 69]. The presence of the B7-H5 protein expression has been demonstrated in gastrointestinal cancer [70-76], gastric cancer [74], lung cancer [7], pancreatic cancer [7, 75], myeloma [76] and head and neck cancer [72]. B7-H5 protein level in OSCC was higher than in normal mucosa and correlated with nodal metastases. B7-H5 expression was not an independent prognostic factor and was not associated with poor prognosis. The high level of VISTA was significantly correlated with PD-L1, CTLA-4, IL13Rα2 (interleukin-13 receptor subunit α2), PI-3K, p-STAT3 (signal transducer and activator of transcription 3), CD11b (integrin αb) and CD33 (siglec-3/sialic acid binding Ig-like lectin 3) protein expression. High levels of VISTA and low levels of CD8 were associated with poor prognosis in the primary OSCC. These results indicate that VISTA may be a potential immunotherapeutic target in OSCC [19].

B7-H6 and NKp30

B7-H6 protein is a type I membrane protein encoded by the *B7-H6* gene. This molecule has two domains: IgC and IgV. It binds to NKp30 molecule. B7-H6 protein was not found in healthy tissue, but was found on human monocytes and macrophages after LPS (lipopolysaccharides) and IFN-g stimulation. It was not observed on B or T cells. It is responsible for the activation of the immune system [7, 77]. The presence of the B7-H6 protein expression has been demonstrated in lung cancer [78], ovarian cancer [79], hepatocellular cancer [80], glioma [81], leukaemia, various sarcomas and renal cancer [82]. The literature documenting B7-H6 protein in OSCC is limited. In one of the studies, the percentage of common B7-H6 protein range was 48% and correlated with grade, overall survival and disease-free survival. The recurrence, differentiation and expression of B7-H6 protein were related to the prognosis [20].

NKp30 receptor is binding to B7-H6 protein. It is a type I protein encoded by the *NCR3 I* gene. NKp30 belongs to NCR family proteins (natural cytotoxicity receptors). This receptor is found on NK cells. NKp30 consists of the ectodomains: a membrane proximal stalk domain and a distal ligand-binding domain. The stalk domain can be important for binding to ligands. It has three isoforms: NKp30a, NKp30b and NKp30c. NKp30a and NKp30b have a stimulating effect and NKp30c has a suppressive effect. NKp30 recognizes B7-H6 antigens on the tumour. In a properly functioning system, NK cells destroy cancer cells directly *via* cytotoxicity or indirectly by cytokine secretion. The glycosylation status of NKp30 alters its binding affinity for B7-H6 [7, 83]. Expression of NKp30 protein was demonstrated in lung cancer (using flow cytometry) [78].

B7-H7 and CD28H

B7-H7 is encoded by the *HHLA2* gene on chromosome 3 (locus 3q13.13). B7-H7 is a co-stimulation protein with three immunoglobulin-like domains (IgV-IgC-IgV). In contrast to other B7 family proteins, the expression of this protein is very low in healthy cells. It is present on monocytes, macrophages, B lymphocytes, mature dendritic cells, endothelial cells, epithelial cells and syncytiotrophoblast cells. There are probably two receptors of the B7-H7 – CD28H molecule with opposite functions. Binding of antigen-presenting cells (APC) to CD28H stimulates T cell proliferation and cytokine production. B7-H7 is likely to have a greater effect on NK cells (*via* the NKp30 receptor) than on T cells (interferon-γ, TNF-α, IL-5, IL-10, IL-13, IL-17a, and IL-22). The presence of the B7-H7 protein expression has been demonstrated in various cancer cell lines: leukaemia, neuroblastoma, mela-

noma, ovarian cancer, breast cancer, lung cancer, thyroid cancer, oesophagus cancer, gastric cancer, colon cancer, pancreatic cancer, hepatocellular cancer, bladder cancer and renal cancer [7, 8, 84, 85]. The studies were carried out using immunohistochemistry [7, 9]. B7-H7 expression was with poor overall survival.

CD28H is a receptor encoded by the *TMIGD2* gene on chromosome 19 (locus 19p13.3). The TMIGD2 has genes parallel to CD28 receptor in 22.8%. It binds to B7-H7. CD28H is found in natural killer cells, APC cells and naive T lymphocytes in peripheral blood. Binding to the receptor can trigger two actions: stimulation and suppression [7, 8, 85].

Conclusions

The expression status of B7 proteins in OSCC cells remains unclear. There are not many scientific reports. The rapid increase in knowledge about the molecules of the B7/CD28 family opens new possibilities for the treatment and prognosis of patients. The data presented indicate the complexity of cellular and molecular processes related to the formation, development and invasion of oral cancer. Various mutations in genes encoding the B7 family and quantitative and functional changes in B7 proteins may be important in the prognosis of OSCC.

Conflict of interest

The authors declare no conflict of interest.

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