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Die molekularbiologische Funktion von Actin beta-like 2 im Ovarialkarzinom

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Nicole Elisabeth Topalov

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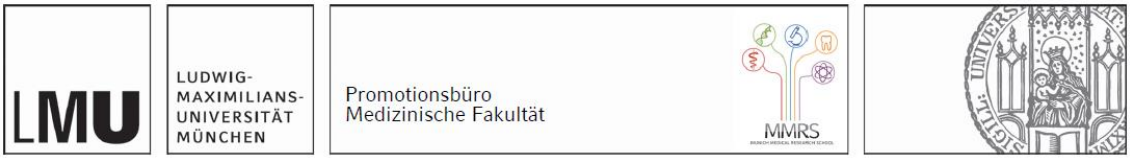

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Inhaltsverzeichnis

Abkürzungsverzeichnis

ACTBL2	Actin beta-like 2
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
BRAF	B-Rapidly Accelerating Fibrosarcoma-Gen
BRCA 1/2	Breast Cancer Antigen 1/2
Cc	Korrelationskoeffizient
CD	Cluster of Differentiation
CPT1	Carnitin-Palmitoyltransferase 1
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
HER2	Human epidermal growth factor receptor 2
HGSOC	High-grade seröses Ovarialkarzinom
HR	Hazard Ratio
HRD	Homologe Rekombinations-Defizienz
KRAS	Kirsten rat sarcoma-Gen
LGSOC	Low-grade seröses Ovarialkarzinom
MIRV	Mirvetuximab-Soravtansin
mRNA	Messenger ribonucleic acid
NFAT5	Nuclear factor of activated T-cells 5
OS	Overall survival
PARPi	Poly-ADP-Ribose-Polymerase-Inhibitor
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand-1
PFS	Progression-free survival
siRNA	Small interfering ribonucleic acid
STIC	Serös tubares intraepitheliales Karzinom
TILs	Tumorinfiltrierende Leukozyten
TMA	Tissue Microarray
TNM	Tumorausbreitung, Lymphknotenbefall und Metastasen

Publikationsliste

Bestandteil der vorliegenden kumulativen Dissertation sind folgende Publikationen:

Topalov NE, Mayr D, Scherer C, Chelariu-Raicu A, Beyer S, Hester A, Kraus F, Zheng M, Kaltofen T, Kolben T, Burges A, Mahner S, Trillsch F, Jeschke U, Czogalla B.

Actin Beta-Like 2 as a New Mediator of Proliferation and Migration in Epithelial Ovarian Cancer.
Front Oncol. 2021 Sep 23;11:713026. doi: 10.3389/fonc.2021.713026.

und

Topalov NE, Mayr D, Kuhn C, Leutbecher A, Scherer C, Kraus FBT, Tauber CV, Beyer S, Meister S, Hester A, Kolben T, Burges A, Mahner S, Trillsch F, Kessler M, Jeschke U, Czogalla B.

Characterization and prognostic impact of ACTBL2-positive tumor-infiltrating leukocytes in epithelial ovarian cancer.
Sci Rep. 2023 Dec 18;13(1):22620. doi: 10.1038/s41598-023-49286-9.

Weitere Publikationen als Co-Autorin:

1. Kraus FBT, **Topalov NE**, Deuster E, Hysenaj I, Mayr D, Chelariu-Raicu A, Beyer S, Kolben T, Burges A, Mahner S, Trillsch F, Jeschke U, Czogalla B.
   Expression pattern and prognostic potential of histamine receptors in epithelial ovarian cancer.
   J Cancer Res Clin Oncol. 2022 Jun 25. doi: 10.1007/s00432-022-04114-x.

2. Beyer S, Müller L, Mitter S, Keilmann L, Meister S, Buschmann C, Kraus F, **Topalov NE**, Czogalla B, Trillsch F, Burges A, Mahner S, Schmoeckel E, Löb S, Corradini S, Kessler M, Jeschke U, Kolben T.
   High RIG-I and EFTUD2 expression predicts poor survival in endometrial cancer.
   J Cancer Res Clin Oncol. 2022 Sep 7. doi: 10.1007/s00432-022-04271-z.

3. Liao Y, Badmann S, Kraus F, **Topalov NE**, Mayr D, Kolben T, Hester A, Beyer S, Mahner S, Jeschke U, Trillsch F, Czogalla B, Burges A.
   PLA2G7/PAF-AH as Potential Negative Regulator of the Wnt Signaling Pathway Mediates Protective Effects in BRCA1 Mutant Breast Cancer.
   Int J Mol Sci. 2023 Jan 3;24(1):882. doi: 10.3390/ijms24010882.

4. Vogelsang TLR, Kast V, Bagnjuk K, Eubler K, Jeevanandan SP, Schmoeckel E, Trebo A, **Topalov NE**, Mahner S, Mayr D, Mayerhofer A, Jeschke U, Vattai A.
   RIPK1 and RIPK3 are positive prognosticators for cervical cancer patients and C2 ceramide can inhibit tumor cell proliferation in vitro.
   Front Oncol. 2023 May 1;13:1110939. doi: 10.3389/fonc.2023.1110939.

5. Reichenbach J, Fraungruber P, Mayr D, Buschmann C, Kraus FBT, **Topalov NE**, Chelariu-Raicu A, Kolben T, Burges A, Mahner S, Kessler M, Jeschke U, Czogalla B, Trillsch F.
   Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer.
   J Cancer Res Clin Oncol. 2023 Sep;149(11):8719-8728. doi: 10.1007/s00432-023-04708-z.

6. Vogelsang TLR, Schmoeckel E, **Topalov NE**, Ganster F, Mahner S, Jeschke U, Vattai A.
   Prognostic Impact of Heat Shock Protein 90 Expression in Women Diagnosed with Cervical Cancer.
   Int J Mol Sci. 2024 Jan 26;25(3):1571. doi: 10.3390/ijms25031571.

Darüber hinaus wurden publizierte Ergebnisse im Rahmen von Posterbeiträgen auf folgenden nationalen Fachkonferenzen vorgestellt:

1. **Topalov NE**, Mayr D, Scherer C, Chelariu-Raicu A, Beyer S, Hester A, Kraus FBT, Zheng M, Kaltofen T, Kolben T, Burges A, Mahner S, Trillsch F, Jeschke U und Czogalla B.
   Die funktionelle Rolle von Actin beta-like 2 in der Pathogenese des Ovarialkarzinoms und dessen prognostische Bedeutung für das Gesamtüberleben.
   96. Kongress der Bayerischen Gesellschaft für Geburtshilfe und Frauenheilkunde e.V. (BGGF), Würzburg, Deutschland, 19. – 21. Mai 2022 in der Postersitzung Gynäkologische Onkologie.

2. **Topalov NE**, Mayr D, Kuhn C, Leutbecher A, Scherer C, Kraus FBT, Beyer S, Meister S, Hester A, Buschmann C, Kolben T, Burges A, Mahner S, Trillsch F, Jeschke U und Czogalla B.
   Der prognostische Einfluss von ACTBL2-exprimierenden tumorinfiltrierenden Leukozyten auf das Überleben von Ovarialkarzinompatientinnen.
   64. Kongress der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe e.V. (DGGG), München, Deutschland, 12.–15. Oktober 2022 in der Sitzung Gynäkologische Onkologie VI – Auszeichnung mit dem Posterpreis der DGGG.

3. Vogelsang TLR, Kast V, Bagnjuk K, Eubler K, Jeevandan SP, Schmoeckel E, Trebo A, **Topalov NE**, Mahner S, Mayr D, Mayerhofer A, Jeschke U, Vattai A.
   RIPK1 und RIPK3 sind positive Prognostikfaktoren für Patientinnen mit Zervixkarzinom und C2 Ceramid inhibiert die Tumorproliferation in vitro.
   Gemeinsamer Kongress der Bayerischen und Österreichischen Gesellschaft für Gynäkologie und Geburtshilfe e.V., Würzburg, Deutschland, 23.–24. Juni 2023 in der Sitzung Gynäkologische Onkologie – Auszeichnung mit dem 1. Posterpreis als Vortragende.

Beitrag zu den Publikationen

Beitrag zu Publikation I

Topalov NE, Mayr D, Scherer C, Chelariu-Raicu A, Beyer S, Hester A, Kraus F, Zheng M, Kaltofen T, Kolben T, Burges A, Mahner S, Trillsch F, Jeschke U, Czogalla B.

Actin Beta-Like 2 as a New Mediator of Proliferation and Migration in Epithelial Ovarian Cancer.

Front Oncol. 2021 Sep 23;11:713026. doi: 10.3389/fonc.2021.713026.

Als Erstautorin dieser Publikation war ich neben meinem Doktorvater Prof. Dr. rer. nat. Udo Jeschke und meinem Betreuer PD Dr. med. Bastian Czogalla maßgeblich an der Konzeptualisierung des Projekts beteiligt. Der überwiegende Großteil der dargelegten Experimente wurde von mir selbstständig und eigenverantwortlich durchgeführt. Lediglich die Analyse anhand der Kaplan-Meier-Plotter- und TIMER-Database erfolgte durch den Co-Autor Mingjun Zheng. Die statistische Auswertung der Überlebens-, Korrelations- und Regressionsanalysen mittels SPSS erfolgte gemeinsam mit Prof. Dr. rer. nat. Udo Jeschke. Die restlichen statistischen Analysen habe ich eigenständig durchgeführt. Zudem habe ich alle gezeigten Grafiken, Tabellen und Fotografien selbst angefertigt und war verantwortlich für die Visualisierung der Ergebnisse. Das Manuskript habe ich, nach Rücksprache bezüglich der Gliederung und der kohärenten Darlegung der Ergebnisse mit PD Dr. med. Bastian Czogalla, vollständig allein ausgearbeitet und verfasst. Die finale Version des Manuskripts wurde primär durch PD Dr. med. Bastian Czogalla und im Anschluss durch alle oben genannten Co-Autor:innen evaluiert. Bei der Einreichung des Manuskripts bei *Frontiers in Oncology* wurde ich durch PD Dr. med. Bastian Czogalla und während der sich anschließenden Revision zusätzlich durch Prof. Dr. rer. nat. Udo Jeschke unterstützt.

Beitrag zu Publikation II

Topalov NE, Mayr D, Kuhn C, Leutbecher A, Scherer C, Kraus FBT, Tauber CV, Beyer S, Meister S, Hester A, Kolben T, Burges A, Mahner S, Trillsch F, Kessler M, Jeschke U, Czogalla B.

Characterization and prognostic impact of ACTBL2-positive tumor-infiltrating leukocytes in epithelial ovarian cancer.
Sci Rep. 2023 Dec 18;13(1):22620. doi: 10.1038/s41598-023-49286-9.

Bei dieser Publikation war ich als Erstautorin gemeinsam mit Prof. Dr. rer. nat. Udo Jeschke und PD Dr. med. Bastian Czogalla maßgeblich verantwortlich für die Ideenfindung und Versuchsplanung. Hierbei wurden wir zusätzlich von Prof. Dr. med. Doris Mayr in ihrer Funktion als Gynäkopathologin unterstützt. Alle in dieser Publikation dargelegten Versuche wurden von mir eigenständig durchgeführt und anschließend ausgewertet. Sämtliche Abbildungen, Fotografien und Grafiken habe ich selbstständig angefertigt und zusammengestellt und war somit federführend in der Visualisierung und Analyse der Daten. Die statistische Auswertung habe ich unter der Supervision von Prof. Dr. rer. nat. Udo Jeschke ebenfalls selbst durchgeführt und ausgearbeitet. Das Manuskript habe ich vollständig allein verfasst. Im Anschluss wurde die primäre Version zunächst von PD Dr. med. Bastian Czogalla und danach durch alle oben genannten Co-Autor:innen evaluiert. Sowohl das Einreichen des Manuskripts bei *Scientific Reports* als auch die sich anschließende Revision habe ich in Rücksprache mit PD Dr. med. Bastian Czogalla eigenverantwortlich durchgeführt.

Einleitung

1 Das Ovarialkarzinom

1.1 Epidemiologie

Das Ovarialkarzinom ist das fünfthäufigste Malignom der Frau in Deutschland und die tödlichste gynäkologische Tumorentität weltweit [1, 2]. Fünf Prozent aller Krebssterbefälle sind hierzulande auf diese Erkrankung zurückzuführen [1]. Im Jahr 2020 erkrankten in Deutschland 7180 Frauen neu an einem Ovarialkarzinom mit einem mittleren Alter von 68 Jahren, wobei das Risiko bis zum 85. Lebensjahr kontinuierlich ansteigt [1]. Weitere Risikofaktoren sind ein erhöhter Body-Mass-Index, Nulliparität sowie genetische Prädispositionen wie eine Mutation im BRCA-Gen [1, 3]. Trägerinnen einer BRCA1-Mutation haben bis zum 69. Lebensjahr ein kumulatives Risiko von schätzungsweise 39% an einem Ovarialkarzinom zu erkranken, Frauen mit einer BRCA2-Mutation lediglich von 11-22%, [4]. Eine signifikante Risikoreduktion unabhängig vom Mutationsstatus kann durch die Einnahme oraler Kontrazeptiva erreicht werden, da die Ovulationshemmung einen protektiven Effekt aufweist [1, 4, 5]. Bisher konnte durch Screeningverfahren mittels Tumormarker-Bestimmung von CA125 und transvaginaler Sonografie keine signifikante Reduktion der Mortalität nachgewiesen werden, weshalb ein standardisiertes Früherkennungsprogramm nicht existiert und eine routinemäßige Durchführung dieser Verfahren nicht empfohlen wird [4, 6]. Zu den prognostischen Faktoren gehören unter anderem das Alter der Patientin bei Erstdiagose, der histologische Subtyp sowie das vorliegende FIGO-Stadium, wobei der postoperative Tumorrest entscheidend ist [4, 7-9]. Das relative 5-Jahres-Überleben liegt über alle Stadien hinweg bei lediglich 43% [4].

1.2 Ätiologie und Pathohistologie

Über 95% aller ovariellen Malignome sind epithelialen Ursprungs [10]. Die restlichen 5% entfallen auf die heterogene Gruppe der nicht-epithelialen Tumore bestehend aus Keimzell- und Keimstrang-Stroma-Tumoren sowie ovariellen Sarkomen [11]. Histopathologisch werden fünf verschiedene Subtypen des Ovarialkarzinoms differenziert, welche sich hinsichtlich ihrer Vorläuferläsionen und molekulargenetischen Faktoren grundlegend unterscheiden: high-grade serös, low-grade serös, endometrioid, muzinös und klarzellig [10]. Hierbei bildet das high-grade seröse Ovarialkarzinom (HGSOC) mit ca. 70% die mit Abstand größte Subgruppe und ist am häufigsten mit einer BRCA1/2-Mutation assoziiert [4, 10]. Dessen Ursprung liegt nach letzten Erkenntnissen in der distalen Tube im Bereich der Fimbrien oder des Infundibulums als sogenanntes seröses tubares intraepitheliales Karzinom (STIC), welches die früheste Manifestation des high-grade

serösen Ovarialkarzinoms darstellt [4, 12]. Ein dualistisches Modell der Ätiopathogenese epithelialer Ovarialtumore postuliert zwei verschiedene Entstehungsmechanismen und unterscheidet Typ-I und Typ-II Karzinome (Tabelle 1) [13]. Typ-I Karzinome, zu denen u.a. das low-grade seröse Ovarialkarzinom (LGSOC) zählt, weisen gehäuft Alterationen im KRAS/BRAF-Signalweg auf und entwickeln sich meist über einen längeren Zeitraum über benigne Neoplasien oder auf dem Boden von Borderline-Tumoren [13-15]. Im Gegensatz dazu entstehen Typ-II Karzinome wie das high-grade seröse Ovarialkarzinom überwiegend *de novo* und zeichnen sich durch eine hohe genetische Instabilität und eine Mutation im Tumorsuppressorgen *p53* aus, welches sich klinisch in einem aggressiveren Wachstum durch eine erhöhte Proliferationsrate manifestiert [13, 15].

Typ-I Karzinom	Typ-II Karzinom
low-grade serös, muzinös, endometrioid oder klarzellig	high-grade serös, Karzinosarkom, undifferenziertes Karzinom
genetisch stabil	genetisch instabil
Mutationen: KRAS/BRAF, β-Catenin, PTEN, ErbB2	Mutationen: p53, BRCA1/2,
Entwicklung über benigne Vorläuferläsionen wie Zystadenome oder Borderline-Tumore	de novo Entstehung, STIC

Tabelle 1: Übersicht der Charakteristika von Typ-I und -II Karzinomen [13, 15].

1.3 Klinik, Diagnostik und Stadieneinteilung

Die Stadieneinteilung des Ovarialkarzinoms erfolgt nach der international und allgemein gültigen FIGO-Klassifikation, welche zuletzt im Jahr 2014 überarbeitet wurde [4]. Aufgrund überwiegend unspezifischer Symptome und einem bis dato fehlenden, suffizienten Screening-Programm wird die Erkrankung in 73% Prozent der Fälle erst in einem fortgeschrittenen Stadium mit zumeist bereits peritonealer Metastasierung (≥ FIGO III) erkannt [1]. Zu den klinischen Manifestationen zählen eine isolierte Bauchumfangsvermehrung bedingt durch Aszites, Verdauungsbeschwerden sowie unspezifische abdominelle Schmerzen [4, 16]. Diagnostisch werden zunächst eine gynäkologische Untersuchung und eine transvaginale Sonografie durchgeführt. Bei sich erhärtendem Verdacht schließt sich eine weiterführende Bildgebung an, bestehend aus einer Computertomografie des Abdomen und des Thorax, da bei 21% der Patientinnen bei Erstdiagnose bereits eine Fernmetastasierung vorliegt [1]. Fehlt selbige, ist das operative Staging mittels

explorativer Laparotomie mit dem Ziel der makroskopischen Tumorfreiheit entscheidend für die Stadieneinteilung und die Prognose [4]. Nach erfolgter histologischer Sicherung und abgeschlossenem Staging wird zudem jeder Patientin eine genetische Beratung und Untersuchung zur Ermittlung des BRCA1/2- sowie des HRD-Status empfohlen [4, 17]. Hiernach wird dann unter Berücksichtigung aller Faktoren das jeweilige Therapiekonzept der Patientin festgelegt.

1.4 Therapie

Die Therapie des Ovarialkarzinoms richtet sich nach dem jeweils vorliegenden FIGO Stadium. Das Staging erfolgt operativ mittels explorativer Längslaparotomie zur Festlegung der initialen Ausbreitung [4]. Der wichtigste prognostische Faktor hierbei ist der intraoperative Tumorrest bzw. die makroskopische Tumorfreiheit nach primärer zytoreduktiver Operation [9]. Zur Gewährleistung einer optimalen Therapie mit bestmöglicher operativer Expertise sollte diese in zertifizierten gynäkoonkologischen Zentren erfolgen [18]. In der Regel schließt sich eine adjuvante platinhaltige Chemotherapie bestehend aus sechs Zyklen Carboplatin in Kombination mit Paclitaxel an [9]. Ein Fertilitätserhalt bzw. Verzicht auf eine adjuvante Systemtherapie sollte nur in einem sehr frühen Stadium bei lediglich unilateralem Befall des Ovars (FIGO IA1) nach ausführlicher Risiko-Nutzen-Aufklärung in Erwägung gezogen werden [4]. In einem fortgeschrittenen Stadium (≥FIGO III) wird zusätzlich eine zielgerichtete Erhaltungstherapie empfohlen [4]. Diese erfolgt je nach Risikoprofil der Patientin unter anderem durch die Hinzunahme eines Angiogenese-Inhibitors in Form von Bevacizumab für insgesamt 15 Monate zur platinbasierten Standard-Chemotherapie [4, 19, 20]. Fester Bestandteil im Rahmen der Diagnostik und Planung der Therapiestrategie ist eine genetische Beratung und Testung der Patientin mit Bestimmung des BRCA- sowie des HRD-Status [4, 17]. Je nach Ergebnis kann in der Erhaltungstherapie auf Poly-ADP-Ribose-Polymerase-Inhibitoren (PARPi) zurückgegriffen werden [4, 21]. Die Zulassung von Olaparib im Jahr 2019 veränderte die Erhaltungstherapie in der Erstlinie maßgeblich. Im Rahmen der SOLO-1 Studie konnte bei Patientinnen mit fortgeschrittenem, BRCA-mutierten Ovarialkarzinom unter Einnahme von Olaparib erstmalig eine hoch signifikante Verbesserung des progressionsfreien Überlebens (PFS) gegenüber einem Placebo mit einer Risikoreduktion für eine Krankheitsprogression oder Tod um 70% gezeigt werden (HR 0,30 [95% CI 0,23–0,41], p<0,001) [4, 22]. Basierend auf den Daten der AGO-OVAR 21/ PRIMA/ ENGOT-ov26-Studie folgte ein Jahr später die Zulassung von Niraparib für das fortgeschrittene Ovarialkarzinom nach Platinansprechen in der Erstlinientherapie unabhängig vom BRCA-Mutationsstatus [23]. Eine kombinierte Erhaltungstherapie mit Olaparib und Bevacizumab führte im Vergleich zu einer Bevacizumab-Monotherapie ebenfalls unabhängig vom BRCA-Mutationsstatus zu einem signifikant verbesserten PFS der untersuchten

Patientinnen [24]. Der stärkste Effekt konnte jedoch im Rahmen der AGO-OVAR 20/ PAOLA-1-Studie in der Subgruppe der BRCA-mutierten Patientinnen oder bei jenen mit einer homologen Rekombinations-Defizienz (HRD-positiv) nachgewiesen werden, wohingegen HRD-negative Patientinnen davon nicht profitierten [24].

Die Therapie der Rezidiverkrankung, welche meist als chronisch anzusehen ist, besteht ebenfalls aus einem multimodalen Konzept. Kommt eine Patientin für eine Rezidivoperation in Frage, so ist das Erreichen einer makroskopischen Tumorfreiheit erneut das oberste Ziel [25]. Im Gegensatz zu anderen gynäkologischen Malignomen spielt die Radiotherapie in der Behandlung des Ovarialkarzinoms keine signifikante Rolle. In Einzelfällen kann in der Rezidivsituation eine lokalisierte Bestrahlung zur Symptomkontrolle nach interdisziplinärer Indikationsstellung in Erwägung gezogen werden [26]. Für die Wahl der Systemtherapie ist das Ansprechen auf die letzte platinhaltige Therapielinie entscheidend. Erkrankt eine Patientin, die auf die platinhaltige Erstlinientherapie angesprochen hat, frühestens sechs Monate nach Therapieabschluss an einem Rezidiv, so kann ihr erneut eine platinhaltige Kombinationstherapie angeboten werden. Bei erfolgreichem Ansprechen auf diese Therapie kann anschließend eine Erhaltungstherapie mit PARPi unabhängig vom BRCA-Mutationsstatus erfolgen [4, 27]. Bei einem platinresistenten Rezidiv stehen platinfreie Monochemotherapien zur Verfügung, jedoch leider nur mit einer Ansprechrate von unter 15% [4, 28]. Neue, zielgerichtete Ansätze versuchen durch den Einsatz von u.a. Antikörper-Drug-Konjugaten diesem Missstand entgegenzutreten. Im Rahmen der MIRASOL-Studie wurde die Wirksamkeit von Mirvetuximab-Soravtansin (MIRV) bei Patientinnen mit einem platinresistenten Ovarialkarzinomrezidiv und einer hohen Folatrezeptor-alpha Expression untersucht. Im Vergleich zur Monochemotherapie konnte durch den Einsatz von MIRV ein signifikanter Benefit der Patientinnen sowohl im progressionsfreien Überleben als auch im Gesamtüberleben (OS) erzielt werden [29]. Trastuzumab-Deruxtecan wiederum könnte bei Patientinnen mit HER2-positivem Ovarialkarzinom zukünftig das Therapiespektrum erweitern und zu einer verbesserten Prognose in der Rezidivsituation beitragen. Erste Erkenntnisse hierzu lieferte die DESTINY-PanTumor02-Studie mit einer signifikanten objektiven Ansprechrate und einem deutlichen Überlebensvorteil der entsprechenden Patientinnen [30].

2 Actin beta-like 2 (ACTBL2)

Actin beta-like 2 (ACTBL2) gilt als eine neu beschriebene Aktin-Isoform des Zytoskeletts, welche zu 92% Strukturgleichheit zu ß-Aktin besitzt [31, 32]. Nichtsdestoweniger zeigen die insgesamt bislang rar gesäten Untersuchungen dieses Proteins deutliche Abweichungen hinsichtlich der molekularbiologischen Funktionen und der intrazellulären

Verteilung im Vergleich zu anderen, deutlich besser verstandenen Aktin-Isoformen [33, 34]. Bisher konnte eine signifikant erhöhte ACTBL2-Expression im kolorektalen Karzinom sowie im duktalen Pankreaskarzinom beschrieben werden [35, 36]. Im hepatozellulären Karzinom war eine hohe ACTBL2-Expression mit einem signifikant schlechteren postoperativen Überleben der betroffenen Patienten assoziiert [37]. Weitere Studien anhand von Zellen des malignen Melanoms konnten ACTBL2 als Bindungspartner von Gelsolin in Lamellipodien identifizieren und so dessen pro-migratorischen Einfluss auf Tumorzellen feststellen [38]. Umgekehrt resultierte eine verminderte Expression von ACTBL2 in einer veränderten Struktur des Zytoskeletts und in ein hierdurch herbeigeführtes, reduziertes zelluläres Invasions- und Migrationsvermögen [33]. Hierzu konkordant konnte ein signifikanter Einfluss auf die zelluläre Motilität anhand von funktionellen Analysen in glatten Muskelzellen dargelegt werden [39].

Eine Exom-Sequenzierungsanalyse konnte *ACTBL2* als putatives Risikogen im Ovarialkarzinom identifizieren [40]. Trotz dessen ist die Bedeutung von Actin beta-like 2 in der Karzinogenese und Pathophysiologie dieser Tumorentität bis dato ungeklärt.

3 Nuclear factor of activated T-cells 5 (NFAT5)

In silico Analysen der Promoter-Region von *ACTBL2* zeigten mehrere Bindungsstellen des Transkriptionsfaktors Nuclear factor of activated T-cells 5 (NFAT5) [39], welcher unter anderem an der Regulation von osmotischem Stress sowie zellulärer Invasion und Migration beteiligt ist [41-44]. Dabei kommt NFAT5 grundsätzlich im Zellkern und im Zytoplasma vor und kann, sowohl abhängig von der extrazellulären Tonizität als auch als Folge von posttranslationalen Modifikationen, in eines der beiden Zellkompartimente gelangen [45-47]. Untersuchungen im hepatozellulären Karzinom zeigten einen signifikanten Überlebensnachteil von Patienten mit einer hohen NFAT5-Expression in den entsprechenden Zellen und konnten ein damit assoziiertes, erhöhtes Rezidivrisiko nachweisen [48]. Ein ähnlicher, statistisch unabhängiger Effekt auf das progressionsfreie Überleben konnte bei Patienten mit nicht-kleinzelligem Bronchialkarzinom nach erfolgter operativer Resektion gezeigt werden [49]. In glatten Muskelzellen resultierte ein gezielter Knockdown von *NFAT5* in einer signifikant verminderten ACTBL2-Expression, welches auf eine direkte Interaktion schließen lässt [39]. Hiervon abgesehen existieren zum jetzigen Zeitpunkt keine Publikationen hinsichtlich des funktionellen Zusammenspiels von Actin beta-like 2 und NFAT5 oder deren Einfluss auf die zelluläre Migration und Proliferation im Ovarialkarzinom.

Darüber hinaus spielt NFAT5 in der Regulation des Tumormikromilieus eine Rolle. Tillé et al. konnten herausarbeiten, dass eine Überexpression von NFAT5 in CD8-positiven T-

Zellen deren tumorsuppressive Funktion drosselte [50]. NFAT5-defiziente Makrophagen hingegen zeigten eine verminderte antitumorale Aktivität gegenüber Ovarialkarzinomzellen *in vivo*, was mit einer reduzierten Ansammlung von T-Effektorzellen im Tumor assoziiert war [51]. Weiterführende Untersuchungen zum Einfluss von NFAT5 auf immunologische Prozesse im Ovarialkarzinom stehen bisher noch aus.

4 Tumorinfiltrierende Leukozyten und Immuntherapie im Ovarialkarzinom

Im Gegensatz zu anderen gynäkologischen Malignomen, wie beispielsweise dem Endometrium- oder Zervixkarzinom, konnte die Immuntherapie bislang noch keinen Einzug in die Therapiestandards des Ovarialkarzinoms halten [52, 53]. Einer der Gründe für ein unzureichendes Ansprechen auf verschiedene immuntherapeutische Ansätze liegt in der vergleichsweise niedrigen Tumormutationslast, die mit der Anzahl an Neoantigenen korreliert [54, 55]. Grundlage für den Einsatz von Checkpoint-Inhibitoren ist die Interaktion der Tumorzelle mit den im Tumormilieu vorhandenen Immunzellen über den von ihnen exprimierten PD-1 Rezeptor und seinem Liganden PD-L1 [56]. Darüber hinaus ist die Zusammensetzung des intratumoralen Immunzellinfiltrats für den Erfolg einer Immuncheckpoint-Blockade von Bedeutung [56]. Der Nachweis von intraepithelialen CD8-positiven Lymphozyten sowie ein hohes Verhältnis von zytotoxischen zu regulatorischen T-Zellen im Tumor zeigten einen prognostischen Vorteil für die entsprechenden Ovarialkarzinompatientinnen [57, 58]. Auch tumorassoziierte Makrophagen tragen abhängig von ihrem Polarisationsstatus (M1 oder M2) und den von ihnen produzierten Zytokinen zur Modulierung des Tumormikromilieus bei [56, 59]. Patientinnen mit einem erhöhten Anteil an pro-inflammatorischen, M1-polarisierten Makrophagen weisen einen signifikanten Vorteil im Gesamtüberleben auf [60, 61]. Zudem scheint Paclitaxel die Polarisation von Makrophagen zugunsten des tumorsuppressiven M1-Phänotyps zu fördern, was seinen therapeutischen Effekt unterstützt [62]. Strickland et al. konnten zeigen, dass BRCA-mutierte Tumore nicht nur eine stärkere PD-L1 Expression, sondern zusätzlich auch eine höhere Zahl an Neoantigenen und CD8-positiven Lymphozyten aufweisen [56, 63].

Basierend auf dieser Evidenzgrundlage scheint der Schlüssel zum Erfolg der Immuntherapie in der Kombination mit bereits bewährten Substanzen zu liegen, um durch synergistische Effekte die Immunogenität des Ovarialkarzinoms zu erhöhen und eine Checkpoint-Blockade zu verbessern. Der Einsatz von Avelumab und Atezolizumab in der Erstlinientherapie konnte in klinischen Phase-III-Studien jeweils keinen signifikanten Einfluss auf das PFS nachweisen [64, 65]. Auch beim platinsensiblen Rezidiv konnte der Einsatz von Atezolizumab in Kombination mit Bevacizumab im Rahmen der

ATALANTE/ENGOT-ov29 – Studie den primären Endpunkt nicht erreichen [66]. Etwas vielversprechender scheint die Kombination aus Checkpoint- und PARP-Inhibitoren zu sein, was aktuell Gegenstand diverser Studienkonzepte ist. Ein Zwischenergebnis der DUO-O/AGO-OVAR 23-Studie zeigt erstmals einen signifikanten Überlebensvorteil im PFS für den kombinierten Einsatz von Durvalumab, Bevacizumab und Olaparib in der Erhaltungstherapie bei HRD-negativen Patientinnen ohne BRCA-Mutation [67]. Erste Ergebnisse der MITO 33-Studie, die den Effekt von Niraparib in Kombination mit Dostarlimab im platinresistenten Rezidiv untersucht, sind aktuell noch ausstehend [68].

Es bleibt insgesamt festzuhalten, dass im Hinblick auf eine angestrebte, zielgerichtete Therapie weitere prädiktive Biomarker von Nöten sind, um sowohl geeignete synergistische Substanzen zu identifizieren als auch spezifische Patientengruppen zu definieren, die von einer Immuntherapie langfristig profitieren.

5 Zielsetzung dieser Arbeit

Trotz Identifikation als putatives Risikogen existierten bisher keine Untersuchungen zu Actin beta-like 2 und dessen Einfluss auf die Pathogenese des Ovarialkarzinoms. Basierend auf Vorarbeiten in anderen Tumorentitäten, die auf eine signifikante Rolle in der zellulären Motilität und Migration hinweisen, soll nun mit dieser Arbeit die prognostische und funktionelle Bedeutung von ACTBL2 im Ovarialkarzinom grundlegend untersucht werden.

Der Hauptfokus der ersten Publikation dieser kumulativen Dissertation liegt auf der Expression und dem Einfluss von ACTBL2 in Ovarialkarzinomzellen. Ein Bestandteil hierbei ist die immunhistochemische Expressionsanalyse von ACTBL2 und seinem Transkriptionsfaktor NFAT5 anhand von Tissue Microarrays (TMAs) eines etablierten Patientinnenkollektivs. Mittels sich anschließender, umfassender Korrelations- und Überlebensanalysen soll geklärt werden, inwiefern Actin beta-like 2 als unabhängiger prognostischer Faktor für das Überleben von Ovarialkarzinompatientinnen fungieren kann. Bis dato gibt es keine Evidenz zur Expression von *ACTBL2* und *NFAT5 in vitro*, weshalb zunächst die Basalexpression beider Gene anhand von Zelllinien verschiedener histologischer Subtypen untersucht werden soll. Hieran anknüpfend gilt es durch Western Blot und immunzytochemische Färbungen die Expression auf Proteinebene und vor allem das jeweilige Expressionsmuster darzustellen. Nachdem Arbeiten zu glatten Muskelzellen und Zellen des malignen Melanoms einen signifikanten Einfluss auf deren Motilität beschrieben, soll mittels funktioneller Assays nach gezieltem siRNA Knockdown von *ACTBL2* und *NFAT5* herausgearbeitet werden, ob und in welchem Ausmaß die zelluläre

Migration, Proliferation und Viabilität beeinflusst und inwiefern Rückschlüsse über die Interaktion zwischen ACTBL2 und NFAT5 gezogen werden können. Hieraus sollen neue Erkenntnisse über die Ätiologie des Ovarialkarzinoms sowie hinsichtlich möglicher therapeutischer Ansatzpunkte gewonnen werden.

In der zweiten Publikation rückt die Expression von ACTBL2 in tumorinfiltrierenden Leukozyten (TILs) im Ovarialkarzinom in den Mittelpunkt. Besonders in Anbetracht der aktuellen Studienlage zur Wirksamkeit von Immuntherapien ist es von fundamentaler Bedeutung, neue Prädiktionsfaktoren für ein Ansprechen zu definieren und ein besseres Verständnis der spezifischen Tumorbiologie anzustreben. Angesichts der postulierten promigratorischen Funktion von ACTBL2 als Bestandteil von spezifischen Zellausläufern wie Lamellipodien und Invadopodien [33, 38] soll erstmals untersucht werden, ob und inwiefern ACTBL2 in tumorinfiltrierenden Leukozyten und somit in Zellen mit hohem Migrationspotenzial exprimiert wird. Durch umfassende immunhistochemische Expressionsanalysen und spezifische Immunfluoreszenz-Doppelfärbungen sollen ACTBL2-positive Leukozyten weiter charakterisiert und deren prognostischer Einfluss im Hinblick auf tumorimmunologische Prozesse untersucht werden.

Zusammenfassung

Das Ovarialkarzinom zeichnet sich unter den gynäkologischen Malignomen sowohl durch die höchste Letalität als auch durch die geringste Ansprechrate auf Immuntherapien aus [2, 56]. Trotz intensiver Forschungsbemühungen in den letzten Jahrzehnten existieren bis heute keine validen prognostischen Biomarker, die zur Früherkennung herangezogen werden können, weshalb die Erkrankung zumeist in einem sehr fortgeschrittenen Stadium erkannt wird [4]. Die aktuellen Therapiestandards berücksichtigen die heterogene Biologie der verschiedenen histologischen Subtypen nur geringfügig, sodass im Hinblick auf eine zeitgemäße, zielgerichtete und möglichst individualisierte Therapie neue molekularbiologische Mechanismen in der Pathogenese des Ovarialkarzinoms entschlüsselt werden müssen. Im Rahmen dieser kumulativen Dissertation wurde erstmals grundlegend untersucht, inwiefern Actin beta-like 2 als neu beschriebene Aktin-Isoform eine prognostische und auch funktionelle Rolle in der Ätiologie des Ovarialkarzinoms spielt.

Im Rahmen von immunhistochemischen Expressionsanalysen anhand von Tissue Microarrays von 156 Ovarialkarzinompatientinnen konnten wir nachweisen, dass eine positive ACTBL2-Expression in den Tumorzellen mit einem signifikant schlechteren Gesamtüberleben assoziiert ist (p=0,035). Dabei zeigten high-grade seröse Karzinome einen signifikant höheren ACTBL2-Gehalt im Vergleich zu anderen histologischen Subtypen (Cc=0,253, p=0,003). Mit einer Hazard Ratio von 2,034 konnte die positive, und damit kombinierte zytoplasmatische und membranöse, Expression von ACTBL2 als statistisch unabhängiger prognostischer Faktor in der Gesamtkohorte identifiziert werden (p=0,013). Konkordant zu den hier dargelegten immunhistochemischen Korrelationsanalysen zeigten die beiden serösen Zelllinien OVCAR3 und UWB1.289 den höchsten mRNA Gehalt an *ACTBL2 in vitro*, wobei im Vergleich zur benignen Zelllinie HOSEpiC die Basalexpression von *ACTBL2* in allen vier untersuchten malignen Zelllinien signifikant höher war (p=0,028). Durch Western Blots und zusätzliche immunzytochemische Färbungen anhand von UWB1.289 Zellen wurde die Expression von ACTBL2 auf Proteinebene nachgewiesen und sowohl in der Membran als auch im Zytoplasma abermals dargestellt. Ein gezielter siRNA Knockdown von *ACTBL2* resultierte in einer signifikant herabgesetzten zellulären Viabilität (p=0,008), Proliferation (p=0,012) und Migration (p=0,012), wodurch der funktionelle Einfluss von ACTBL2 im Ovarialkarzinom erstmals dargelegt werden konnte. Um den regulatorischen Effekt von NFAT5 auf die Expression und Funktion von ACTBL2 zu untersuchen, erfolgten ergänzende Analysen *in vitro*, die die zuvor erhobenen Daten insgesamt widerspiegeln und stützen. In seiner Rolle als Transkriptionsfaktor konnte NFAT5 sowohl im Zellkern als auch im Zytoplasma von UWB1.289 Zellen identifiziert werden. Nach erfolgreichem siRNA Knockdown von *NFAT5* zeigte sich eine signifikante Herabsetzung der mRNA Expression von *ACTBL2*

um 46% (p=0,008), was auf eine direkte Interaktion schließen lässt. Konsekutiv durchgeführte funktionelle Assays konnten konkordant zur den Ergebnissen nach primärem *ACTBL2*-Knockdown eine signifikante Reduktion der zellulären Proliferation (p=0,001) und Migration (p=0,012) nachweisen. Ergänzende immunhistochemische Expressionsanalysen von NFAT5 in TMAs zeigten eine signifikante Korrelation zwischen zytoplasmatischer NFAT5-Expression und niedrigem FIGO-Stadium (p=0,022) und Grading (p<0,001), sodass ein Zusammenhang zwischen der vorwiegend transkriptionell inaktiven Form von NFAT5 mit prognostisch günstigen histopathologischen Charakteristika vermutet werden kann.

In 77% aller auswertbaren Fälle zeigten Patientinnen des untersuchten TMA-Kollektivs über alle histologischen Subtypen hinweg eine Tumorinfiltration mit ACTBL2-positiven Leukozyten. Dabei korrelierte deren Nachweis signifikant mit Karzinomen low-grade seröser Histologie (Cc=0,200, p=0,025). In der Gesamtkohorte zeigte sich ein signifikanter Überlebensvorteil von Patientinnen mit einem Nachweis von ACTBL2-exprimierenden Leukozyten (p=0,006). Basierend auf der dargelegten Korrelation wurde dieser prognostische Einfluss in einer Subgruppenanalyse erneut untersucht und ein hochsignifikanter Benefit für Patientinnen mit LGSOC und ACTBL2-positiven TILs herausgearbeitet (p<0,001). In einer multivariaten Analyse konnte deren intratumoraler Nachweis sowohl in der Gesamtkohorte (HR=0,556, p=0,038) als auch in der LGSOC-Subgruppe (HR=0,058, p=0,018) als statistisch unabhängiger prognostischer Faktor für das Gesamtübeleben identifiziert werden. In der Annahme, dass ACTBL2 eine Schlüsselrolle in der Migration von aktivierten Leukozyten spielt, wurde die Co-Expression von CD44 als Adhäsionsmolekül und Mediator von Leukozyteninvasion [69, 70] evaluiert und unabhängig vom histologischen Subtyp mittels Doppel-Immunfluoreszenz nachgewiesen. Zur weiteren Klassifikation der ACTBL2-positiven TILs mit besonderem Augenmerk auf potenziell immunvermittelte, antitumorale Effekte wurden umfassende immunhistochemische Färbungen anhand von eigens angefertigten Serienschnitten durchgeführt, die CD8-positive zytotoxische T-Zellen und CD68-positive Makrophagen als die vorherrschenden Subtypen identifizierten.

Zusammenfassend konnte mit dieser Dissertation erstmals ein signifikanter prognostischer und funktioneller Einfluss von Actin beta-like 2 im Ovarialkarzinom nachgewiesen werden. Insbesondere die dargelegte membranöse Expression von ACTBL2 in Tumorzellen kann vor dem Hintergrund eines pro-migratorischen Effekts von Bedeutung sein, da Analysen anderer Arbeitsgruppen ACTBL2 als Bindungspartner von Gelsolin als Aktin-modulierendes Protein in Lamellipodien ermitteln konnten [38]. Als Mediator zellulärer Proliferation und Migration *in vitro* spiegelt sich die somit postulierte Funktion von Actin beta-like 2 im signifikant schlechteren Gesamtüberleben von Ovarialkarzinompatientinnen mit positiver ACTBL2-Expression wider. Darüber hinaus wurde die modulierende Wirkung von NFAT5 auf die Expression von ACTBL2 und auf die daraus

resultierenden Veränderungen zellulärer Mechanismen zum ersten Mal im Ovarialkarzinom belegt und als putativer Ansatzpunkt für neue, antiproliferative Therapien identifiziert. Ein signifikanter, prognostisch günstiger Einfluss auf das Gesamtüberleben von Ovarialkarzinompatientinnen wiederum konnte ACTBL2-exprimierenden Leukozyten zugeschrieben werden. Die hier dargelegten Ergebnisse deuten darauf hin, dass dieser Effekt auf der Zusammensetzung der Immunzellinfiltrate aus CD44-exprimierenden, zytotoxischen T-Zellen und Makrophagen beruht. Abschließend liefern die Ergebnisse dieses Promotionsprojekts erstmalig Hinweise auf ein Zusammenspiel aus CD44-vermittelten Prozessen in der Leukozytenaktivierung und ACTBL2-abhängigen, pro-migratorischen Veränderungen des Zytoskeletts und können somit als Basis für zukünftige Subgruppenanalysen hinsichtlich der Wirksamkeit von immuntherapeutischen Ansätzen herangezogen werden.

Summary

Among gynaecological malignancies, ovarian carcinoma is distinguished by both the highest mortality rate and the lowest response rate to immunotherapy [2, 53]. Despite intensive research efforts in recent decades, there are still no valid prognostic biomarkers that can be used for early detection, which is why the disease is typically diagnosed at a very advanced stage [4]. The current treatment standards only consider the heterogeneous biology of the various histological subtypes to a limited extent. Consequently, new molecular biological mechanisms in the pathogenesis of ovarian cancer must be identified in order to develop modern, targeted and individualised treatments. In this dissertation, the extent to which actin beta-like 2, a newly described actin isoform, plays a prognostic and functional role in the aetiology of epithelial ovarian cancer was investigated for the first time.

In immunohistochemical expression analyses using tissue microarrays from 156 ovarian cancer patients, we could demonstrate that positive ACTBL2-expression in tumour cells is associated with significantly poorer overall survival (OS) (p=0.035). High-grade serous carcinomas showed a significantly higher ACTBL2-content compared to other histological subtypes (Cc=0.253, p=0.003). With a hazard ratio of 2.034, the positive, and thus combined cytoplasmic and membranous expression of ACTBL2 was identified as a statistically independent prognostic factor for the overall cohort (p=0.013). Consistent with the immunohistochemical correlation analyses presented here, the two serous cell lines OVCAR3 and UWB1.289 showed the highest mRNA content of *ACTBL2 in vitro*, whereby the basal expression of *ACTBL2* was significantly higher in all four malignant cell lines examined compared to the benign cell line HOSEpiC (p=0.028). Western blots and additional immunocytochemical staining using UWB1.289 cells were employed to detect the expression of ACTBL2 at the protein level, with the results visualised both in the membrane and in the cytoplasm. Targeted siRNA-mediated knockdown of *ACTBL2* resulted in a significant reduction in cellular viability (p=0.008), proliferation (p=0.012) and migration (p=0.012), demonstrating the functional influence of ACTBL2 in epithelial ovarian cancer for the first time. In order to investigate the regulatory impact of NFAT5 on the expression and function of ACTBL2, additional *in vitro* analyses were carried out, which reflect and support the previously collected data. In its role as a transcription factor, NFAT5 was detected both in the nucleus and in the cytoplasm of UWB1.289 cells. After successful siRNA knockdown of *NFAT5*, mRNA expression of *ACTBL2* was significantly reduced by 46% (p=0.008), suggesting a direct interaction. Consecutive functional assays showed a significant reduction in cellular proliferation (p=0.001) and migration (p=0.012) concordant with the results after primary *ACTBL2* knockdown. Supplementary immunohistochemical expression analyses of NFAT5 in TMAs showed a significant

correlation between cytoplasmic NFAT5 expression and low FIGO stage (p=0.022) and grading (p<0.001), hinting at a correlation between the predominantly transcriptionally inactive form of NFAT5 and prognostically favourable histopathological characteristics.

In 77% of all evaluable cases, patients in the study cohort showed tumour infiltration with ACTBL2-positive leukocytes across all histological subtypes. Their detection correlated significantly with carcinomas of low-grade serous histology (Cc=0.200, p=0.025). In the overall cohort, there was a significant survival benefit for patients with evidence of ACTBL2-expressing leukocytes (p=0.006). Based on the correlation described above, this prognostic influence was re-examined in a subgroup analysis and a significant benefit was identified for patients with LGSOC and ACTBL2-positive TILs (p<0,001). In a multivariate analysis, their intratumoural detection was identified as a statistically independent prognostic factor for OS in both the overall cohort (HR=0,556, p=0,038) and the LGSOC-subgroup (HR=0,058, p=0,018). Assuming that ACTBL2 plays a key role in the migration of activated leukocytes, co-expression of CD44 as an adhesion molecule and mediator of leukocyte invasion [66, 67] was evaluated and detected by double immunofluorescence independent of histological subtype. For further classification of ACTBL2-positive TILs with special focus on potential immune-mediated antineoplastic effects, extensive immunohistochemical staining was performed on custom-made serial sections containing CD8-positive cytotoxic T-cells and CD68-positive macrophages as the predominant subtypes.

In sum, this dissertation was the first to demonstrate a significant prognostic and functional impact of Actin beta-like 2 in epithelial ovarian cancer. In particular, the membranous expression of ACTBL2 in tumour cells may be important in the context of a pro-migratory effect, as analyses by other research groups have identified ACTBL2 as a binding partner of gelsolin as an actin-modulating protein in lamellipodia [35]. The postulated function of Actin beta-like 2 as a mediator of cell proliferation and migration *in vitro* is reflected in the significantly poorer overall survival of ovarian cancer patients with positive ACTBL2-expression. In addition, the modulating effect of NFAT5 on ACTBL2-expression and the resulting alterations in cellular mechanisms were demonstrated and additionally identified as a putative target for innovative anti-proliferative therapies of ovarian cancer. In turn, a significant and prognostically favourable effect on the OS of ovarian cancer patients was attributed to the presence of ACTBL2-expressing leukocytes. The given results suggest that this effect is due to the composition of the immune cell infiltrates, consisting of CD44-expressing cytotoxic T-cells and macrophages. Concluding, these findings provide for the first time evidence of an interplay between CD44-mediated processes in leukocyte activation and ACTBL2-dependent pro-migratory changes of the cytoskeleton and can thus be used as a basis for future subgroup analyses regarding the efficacy of immunotherapeutic approaches.

Ausblick

Basierend auf den hier dargelegten Ergebnissen gilt es nun in weiterführenden Untersuchungen die Interaktion von ACTBL2 und NFAT5 genauer zu analysieren. Hierzu rückt besonders Etomoxir als putative therapeutische Substanz in den Fokus. Diverse Studien konnten zeigen, dass die Aktivität und nukleäre Translokation von NFAT5 von posttranslationalen Palmitoylierungs-Prozessen abhängig ist [46, 47, 71]. Eine gezielte und irreversible Inhibition der mitochondrialen Carnitin-Palmitoyltransferase 1 (CPT1) durch die Substanz Etomoxir resultierte konsekutiv in einer signifikant reduzierten zytoplasmatischen ACTBL2-Expression [39, 71]. Zum jetzigen Zeitpunkt existieren keine publizierten Studien zum regulatorischen Einfluss von Etomoxir auf ACTBL2 und NFAT5 im Zusammenhang mit gynäkologischen Karzinomen. Da die Ergebnisse dieses Promotionsprojekts eine signifikante Abnahme des Proliferations- und Migrationsvermögen von Ovarialkarzinomzellen nach spezifischem Knockdown von *NFAT5* und *ACTBL2* belegen, könnte der Einsatz von Etomoxir einen innovativen und zielgerichteten antiproliferativen Therapieansatz darstellen. Gleichzeitig könnte in Anbetracht der hier dargestellten Überlebensdaten eine Reduktion des intrazellulären Gehalts von ACTBL2 zu prognostisch günstigen Veränderungen in der Tumorbiologie führen. Somit sollte das Ziel zukünftiger Arbeiten die Analyse des therapeutischen Potentials von Etomoxir im Ovarialkarzinom sein, auch im Hinblick auf mögliche Kombinationen mit bereits etablierten Substanzen der Erstlinientherapie. Zusätzlich bleibt herauszufinden, inwiefern sich die erzielten Forschungsergebnisse auf andere gynäkologische Tumorentitäten übertragen und erweitern lassen.

Gleichzeitig sollten weitere Projekte den Fokus auf eine detailliertere Subtypisierung der ACTBL2-positiven tumorinfiltrierenden Leukozyten im Ovarialkarzinom lenken. Ein spezielles Augenmerk sollte hierbei auf dem Polarisationsstatus ACTBL2-exprimierender Makrophagen und der Interaktion zwischen CD44 und ACTBL2 in der Formation pro-migratorischer Zellausläufer liegen. Besonders angesichts der vergleichsweise enttäuschenden Studienergebnisse immuntherapeutischer Ansätze ist ein besseres Verständnis des Tumormikromilieus und der Zusammensetzung der Immunzellinfiltrate unabdingbar, um zugrundeliegende Prozesse besser zu erfassen und neue Ansatzpunkte in bestimmten Patientenkollektiven zu definieren. Hierfür könnte Actin beta-like 2 als putativer Marker für aktivierte, migrierende Leukozyten eine vielversprechende Option darstellen und sollte somit Gegenstand weiterer, umfassender experimenteller Arbeiten sein.

Publikation I

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Actin Beta-Like 2 as a New Mediator of Proliferation and Migration in Epithelial Ovarian Cancer.

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Abstract:

The impact of Actin beta-like 2 (ACTBL2), a novel described actin isoform, on epithelial ovarian cancer (EOC) biology has not been investigated so far. In this study, we analyzed the prognostic and functional significance of ACTBL2 and its regulatory element Nuclear factor of activated T-cells 5 (NFAT5). The expression of ACTBL2 and NFAT5 was examined in tissue microarrays of 156 ovarian cancer patients by immunohistochemistry. Aiming to assess the molecular impact of ACTBL2 on cellular characteristics, functional assays were executed in vitro upon siRNA knockdown of *ACTBL2* and *NFAT5*. ACTBL2 expression was identified as an independent negative prognostic factor for overall survival of EOC patients. EOC cell lines showed a significantly increased mRNA and protein level of ACTBL2 compared to the benign control. In vitro analyses upon siRNA knockdown of *ACTBL2* displayed a significantly reduced cellular viability, proliferation and migration. siRNA knockdown of *NFAT5* proved a significant molecular interplay by inducing a downregulation of *ACTBL2* with a thus resulting concordant alteration in cellular functions, predominantly reflected in a decreased migratory potential of EOC cells. Our results provide significant evidence on the negative prognostic impact of ACTBL2 in EOC, suggesting its crucial importance in ovarian carcinogenesis by modulating cellular motility and proliferation.

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Actin Beta-Like 2 as a New Mediator of Proliferation and Migration in Epithelial Ovarian Cancer

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The impact of Actin beta-like 2 (ACTBL2), a novel described actin isoform, on epithelial ovarian cancer (EOC) biology has not been investigated so far. In this study, we analyzed the prognostic and functional significance of ACTBL2 and its regulatory element Nuclear factor of activated T-cells 5 (NFAT5). The expression of ACTBL2 and NFAT5 was examined in tissue microarrays of 156 ovarian cancer patients by immunohistochemistry. Aiming to assess the molecular impact of ACTBL2 on cellular characteristics, functional assays were executed *in vitro* upon siRNA knockdown of *ACTBL2* and *NFAT5*. ACTBL2 expression was identified as an independent negative prognostic factor for overall survival of EOC patients. EOC cell lines showed a significantly increased mRNA and protein level of ACTBL2 compared to the benign control. *In vitro* analyses upon siRNA knockdown of *ACTBL2* displayed a significantly reduced cellular viability, proliferation and migration. siRNA knockdown of *NFAT5* proved a significant molecular interplay by inducing a downregulation of ACTBL2 with a thus resulting concordant alteration in cellular functions, predominantly reflected in a decreased migratory potential of EOC cells. Our results provide significant evidence on the negative prognostic impact of ACTBL2 in EOC, suggesting its crucial importance in ovarian carcinogenesis by modulating cellular motility and proliferation.

Keywords: actin beta-like 2, nuclear factor of activated T-cells 5, epithelial ovarian cancer, prognosis, proliferation, migration

INTRODUCTION

Epithelial ovarian cancer (EOC) is the fifth leading lethal tumor entity in women and the most common cause of death among gynecological cancer patients (1). Due to comparably insufficient screening methods and minor clinical symptoms with a consecutively late diagnosis of advanced tumor stages, EOC is associated with a relatively low 5-year survival rate of less than 45% (2). Established and reliable prognostic factors for overall survival of EOC patients include the disease stage at diagnosis (FIGO), tumor grading, histological subtypes and patient's age, with the volume of residual disease after primary surgery being the most significant one (3–6). First-line therapy consists of cytoreductive surgery and adjuvant platinum-based chemotherapy in the clinical course. This is followed by the use of bevacizumab or poly-ADP-ribose-polymerase inhibitors, as a recent promising therapeutic approach in the maintenance treatment of patients with at least partial response to chemotherapy (7, 8). While other gynecological tumor entities such as endometrial and cervical cancer are comparably prone to respond to immune therapy, no promising prognostic benefit in terms of ovarian cancer treatment has been shown yet (9–12). Despite new emerging therapeutic strategies in the past few years, widely accepted and reliable biomarkers for ovarian cancer are still rare due to lacking profound knowledge on molecular pathological mechanisms enhancing tumor development and progression.

Actin beta-like 2 (ACTBL2), a novel described actin isoform showing 92% structural similarity to ß-actin, was found to be a putative risk gene in ovarian cancer (13–15). Yet, the cellular function of ACTBL2 in EOC and its carcinogenetic impact on gynecological malignancies are thus far unknown. Despite the relatively high structural congruence to ß-actin, phylogenetic analyses revealed a genetic distance from other commonly known isoforms, with ACTBL2 being expressed in different cellular localizations and executing individual molecular functions (16, 17). A significant upregulation of ACTBL2 was yet detected in pancreatic ductal adenocarcinoma and colorectal cancer (18, 19). Moreover, a high abundance of ACTBL2 in hepatocellular carcinoma was associated with altered cellular growth properties and an impaired postoperative disease-free survival of affected patients (16). Mazur et al. identified ACTBL2 as a binding partner of gelsolin in melanoma cells, being part of cellular lamellipodia and thus hinting at its intracellular function and putatively promigratory effect (20). Additionally, functional assays revealed an impaired migration of vascular smooth muscle cells (VSMCs) after gene silencing of *ACTBL2* (21). In silico analyses focusing on the promotor sequence of *ACTBL2* displayed several putative binding sites for Nuclear factor of activated T-cells 5 (NFAT5) (21). Executing its manifold functions as a transcription factor, NFAT5 is required in regulating the expression of genes involved in controlling cellular osmotic stress and in orchestrating cellular migration and proliferation (22–25). Gene knockdown of *NFAT5* in vascular smooth muscle cells resulted in a significantly diminished ACTBL2 expression, proving their direct interaction (21). Apart from studies focusing on promigratory effects in biomechanically activated VSMCs, the regulatory impact of NFAT5 on ACTBL2 in tumor cells and the extent of the consequently provided alterations of cellular functions remain still unknown.

The present study aimed at elucidating the functional role of ACTBL2 and NFAT5 in epithelial ovarian cancer, intentionally assisting to obtain new findings on its etiology with regard to carcinogenetic and disease-promoting mechanisms.

Abbreviations: ACTBL2, Actin beta-like 2; BrdU, 5-bromo-2-deoxyuridine; BRCA1, breast cancer gene 1; cDNA, complementary deoxyribonucleic acid; CI, confidence interval; Cc, correlation coefficient; CPT1, carnitine palmitoyltransferase 1; Ct, cycle threshold; DAB, 3,3'diaminobenzidine; DNA, deoxyribonucleic acid; EOC, epithelial ovarian cancer; FAO, fatty acid oxidation; FBS, fetal bovine serum; FFPE, formalin-fixed and paraffin-embedded; GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase; ICC, immunocytochemistry; IHC, immunohistochemistry; IRS, immunoreactive score; FIGO, International Federation of Gynecology and Obstetrics; LMU, Ludwig-Maximilians-University; mRNA, messenger ribonucleic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MCR, Munich Cancer Registry; NFAT5, Nuclear factor of activated T-cells 5; OD, optical density; OS, overall survival; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RIPA, radioimmunoprecipitation assay; ROC, receiver operating characteristic; RNA, ribonucleic acid ; RT, room temperature; siRNA, small interfering ribonucleic acid; TBS, tris-buffered saline; VSMCs, vascular smooth muscle cells; WHO, World Health Organization.

MATERIAL AND METHODS

Ethical Approval

This study was approved by the Ethics Committee of the Ludwig-Maximilians-University (LMU), Munich, Germany (approval number 227-09, 18-392 and 19-972). All tissue samples used were obtained from material initially utilized for pathological diagnostics from the archives of the LMU, Munich, Germany. The diagnostic procedures were completed before the present study was performed, with the observers being fully blinded to the patients' data during all experimental and statistical analyses. All experiments described were performed respecting the standards of the Declaration of Helsinki (1975).

Patients and Specimens

Tissue microarrays of 156 EOC patients who underwent cytoreductive surgery between 1990 and 2002 at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University in Munich, Germany, were analyzed in the given study (**Table 1**). In previously performed studies regarding the present cohort, various other pathological parameters were investigated, thus enabling the execution of correlation analyses. The clinical data was obtained from the patients' charts with the according follow-up data being received from the Munich Cancer Registry (MCR). Only patients with pathologically validated epithelial ovarian cancer were included, whereas benign as well as borderline tumors were accordingly excluded from the collective. Moreover, none of the considered patients had neoadjuvant chemotherapy in the clinical course. All samples used were formalin-fixed and paraffin-embedded (FFPE) before being examined by gynecological pathologists at the Department of Pathology, LMU, regarding clinical and pathological criteria. The samples were classified into histological subtypes [serous (n=110), clear cell (n=12),

TABLE 1 | Clinicopathological characteristics of ovarian cancer patients considered in this study.

Clinicopathological parameters	n	Percentage (%)
Histology		
serous	110	70.5
clear cell	12	7.7
endometrioid	21	13.5
mucinous	13	8.3
Primary tumor expansion		
TX	1	0.6
T1	40	25.6
T2	18	11.5
T3	97	62.3
Nodal status		
pNX	61	39.1
pN0	43	27.6
pN1	52	33.3
Distant metastasis		
pMX	147	94.2
pM0	3	1.9
pM1	6	3.8
Grading serous		
low	24	21.8
high	80	72.7
Grading endometrioid		
G1	6	28.6
G2	5	23.8
G3	8	38.1
Grading mucinous		
G1	6	46.2
G2	6	46.2
G3	0	0
Grading clear cell		
G3	12	100.0
FIGO		
I	35	22.4
II	10	6.4
III	103	66.0
IV	3	1.9
Age		
≤60 years	83	53.2
>60 years	73	46.8

endometrioid (n=21), mucinous (n=13)] as well as rated by tumor grading, respecting the currently valid WHO classifications. Serous ovarian cancer was divided into low and high grading, while tissue samples of endometrioid histology were graded according to G1 to G3. For mucinous ovarian carcinoma, there is no explicit WHO classification; however, this subtype is often classified into G1 to G3 analogous to endometrioid subtype. Clear cell ovarian cancer was always categorized as G3. Further, staging was performed using the FIGO classification [I (n=35), II (n=10), III (n=103), IV (n=3)], while data on primary tumor extension according to the TNM classification was available in 155 cases showing the following distribution: T1 (n=40), T2 (n=18) and T3 (n=97). Concerning lymph node involvement, data was obtainable in 95 cases [N0 (n=43), N1 (n=52)], whereas data on distant metastasis was only accessible in 9 cases [M0 (n=3), M1 (n=6)]. Information on grading and FIGO stage is missing in 12 respectively 5 cases.

Immunohistochemistry

After dewaxing the formalin-fixed and paraffin-embedded ovarian cancer tissue microarrays in xylol for 20 minutes, the slides were shortly washed in 100% ethanol. Intending to avoid unspecific staining, the endogenous peroxidase was blocked by using 3% H_2O_2 in methanol for 20 minutes, before rehydrating the samples in descending concentrations of ethanol (100%, 70% and 50%) and shortly resting them in distilled water. Next, the slides were put in a pressure cooker filled with boiling sodium citrate buffer (pH=6) consisting of 0.1 M citric acid and 0.1 M sodium citrate and were consecutively heated for 5 minutes. Cooled down, the tissue samples were shortly washed in distilled water and then in phosphate-buffered saline (PBS) twice for 2 minutes each. To prevent an unspecific staining reaction during the course, the slides were incubated with a blocking solution [Reagent 1; ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed, Berlin, Germany] for 5 minutes at room temperature (RT) followed by an overnight incubation of 16 hours at 4°C with the following primary antibodies: anti-ACTBL2, 1:800 dilution in PBS (rabbit IgG, polyclonal, abcam, ab100869), anti-NFAT5, 1:200 dilution in PBS (rabbit IgG, polyclonal, Sigma, HPA069711-100UL). Afterwards, the samples were again washed twice in PBS and subsequently treated with a post block reagent (Reagent 2; ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed, Berlin, Germany) for 20 minutes at RT. After repeating the previously described washing step with PBS, the slides were incubated with an HRP-polymer containing bound anti-mouse as well as anti-rabbit antibodies (Reagent 3; ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed, Berlin, Germany) for 30 minutes. For visualization, 3,3'diaminobenzidine (DAB) and the according substrate buffer (Liquid DAB and Substrate Chromogen System, DAKO, Munich, Germany) were applied on the tissue for 30 seconds (ACTBL2) and 1,5 minutes (NFAT5), respectively. The reaction was stopped by washing the slides in distilled water, followed by a counterstaining with Mayer's acidic hemalum (Waldeck, Münster, Germany). After dehydrating the ovarian cancer tissue in a series of ethanol with ascending concentrations (70%, 96% and 100%), the slides were placed in xylol and finally covered. Kidney and vulva tissue served as negative and positive controls to examine the specificity of the immunoreaction as well as to assess the most suitable dilution of the used primary antibodies (**Figure S1**). Concerning the negative controls, the primary antibodies were each replaced by a specific isotype control antibody (BioGenex, Fremont, CA, USA).

Immunocytochemistry

For immunocytochemistry (ICC) of ACTBL2 and NFAT5, assessing the basal protein expression in ovarian cancer cells, 1×10^6 UWB1.289 cells were seeded on sterile microscope slides

and maintained in culture as described below for 24 hours. After washing with PBS twice for 5 minutes each, the slides were fixed by being placed in 100% ethanol and methanol (1:1) at room temperature (RT) for 15 minutes and were subsequently air dried. Intending to avoid unspecific background staining, the slides were treated with a goat-derived serum (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) for 20 minutes at RT after being rehydrated in PBS for 5 minutes. Next, the slides were incubated overnight for 16 hours at 4°C with the primary antibodies mentioned above in a 1:400 (ACTBL2) respectively 1:50 (NFAT5) dilution. Afterwards, the slides were washed in PBS for 5 minutes followed by a 30 minute incubation with a biotinylated secondary anti-rabbit antibody (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) at RT. Again, the slides were washed in PBS and subsequently treated with an avidin-biotin-peroxidase complex (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes at RT. To finally visualize the staining, chromogen 3-amino-9-ethylcarbazole (AEC^+, DAKO, Hamburg, Germany) was applied for 10 minutes at RT. In order to stop the reaction, the slides were placed in distilled water before being counterstained with Mayer's acidic hemalum (Waldeck, Münster, Germany). After being washed in distilled water, the slides were covered using an aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany).

For ICC of ACTBL2 and NFAT5 after gene silencing, 5×10^4 UWB1.289 cells were seeded in each well of sterile 4-well chamber slides (Lab-Tek II Chamber Slides, Thermo Fisher Scientific, Denmark) and maintained in culture overnight. siRNA knockdown of *ACTBL2* respectively *NFAT5* was performed for 48 hours as explained below, before executing the immunocytochemical staining as previously described.

Staining Evaluation and Statistical Analysis

The examination of all EOC specimens was performed using a Leitz photomicroscope (Wetzlar, Germany) with the immunohistochemical (IHC) staining being analyzed by applying the semi-quantitative immunoreactive score (IRS) (26). The score is calculated by multiplying the percentage of positively stained cells (0=no staining, 1 ≤ 10%, 2 = 11-50%, 3 = 51-80% and 4≥81%) by the predominating optical staining intensity (0=no, 1=weak, 2=moderate, 4=strong). For each staining performed, the immunoreactive score was obtained considering the distinct distribution pattern of the analyzed proteins. As separate scores were calculated for each cellular compartment, NFAT5 staining was assessed in the cytoplasm and the nucleus, whereas ACTBL2 expression was evaluated in the cytoplasm and the cell membrane.

For statistical analyses of all data obtained, IBM SPSS Statistics 26.0 (IBM Corporation, Armonk, NY, USA) was used. Spearman's analysis (27) was performed to calculate bivariate correlations between the examined proteins and clinicopathological data. Further, Kruskal-Wallis-H test (28) was used to assess and compare the distribution of more than two independent samples in the analyzed collective. Overall survival of EOC patients was compared by executing log-rank testing with Kaplan-Meier curves being used for visualization (29). For identification of appropriate cut-off values in survival analyses, a ROC curve analysis was performed, being a reliable and widely accepted method for cut-off point selection (30). The Youden index, being defined as the maximum (sensitivity+specificity-1), was used to ensure the optimal cut-off, maximizing the sum of sensitivity and specificity (31, 32). For multivariate analyses, a Cox regression model of the investigated parameters was established (33). qPCR results were analyzed for statistical significance by using the obtained Ct values and calculating the relative expression by applying the $2^{-\Delta\Delta Ct}$ formula (34). Further *in vitro* experiments were statistically analyzed by performing Wilcoxon test with all *in vitro* analyses being visualized using GraphPad Prism 7.00 (San Diego, CA, USA). For all analyses, p-values ≤0.05 were considered to be statistically significant.

Cell Lines

The human ovarian cancer cell lines ES-2 (clear cell), OVCAR3 (serous), TOV112D (endometrioid) and UWB1.289 (serous, BRCA1 negative) were obtained from ATCC (Rockville, MD, USA) and were maintained in culture using RPMI 1640 GlutaMAX Medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK) in a humified incubator at 37°C and 5% CO2. For reference, the benign human cell line HOSEpiC (ATCC, Rockville, MD, USA) was cultured in Ovarian Epithelial Cell Medium (OEpiCM; ScienCell, Carlsbad, CA, USA) according to the instructions of the company in a humified incubator at 37°C and 5% CO2. All cell lines used in this study were tested negative for mycoplasma in advance.

qPCR

mRNA isolation was executed using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Next, 1µg RNA was converted into cDNA using the MMLV Reverse Transcriptase 1st-strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA). The mRNA expression of both ACTBL2 and NFAT5 was quantified by qPCR applying FastStart Essential DNA Probes Master and gene-specific primers (Roche, Basel, Switzerland, **Table S1**), with their relative expression being subsequently calculated by the 2-ΔΔCt formula using GAPDH as a housekeeping gene.

siRNA Knockdown

UWB1.289 cells were transfected with small interfering RNA (siRNA) for *ACTBL2* and *NFAT5*, respectively (GeneSolution siRNA, Qiagen Sciences, MD, USA; for detailed information on the according sequences, see **Tables S2A, B**), by using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). For reference, a scrambled negative control siRNA (AllStars Negative Control siRNA, Qiagen, Hilden, Germany) was utilized. At first, 2,5×105 UWB1.289 cells/well were seeded on sterile 6-well plates and maintained in culture as described above. After reaching a cell density of 60-70%, the transfection was performed by treating the cells with OptiMEM Reduced Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing the siRNA-Lipofectamine complex. After 48 hours of incubation at 37°C and 5% CO2, the cells were harvested and used

for further experiments. To prove the successful gene silencing, mRNA isolation and qPCR were subsequently executed as outlined above. Immunocytochemistry was applied as previously described to confirm the knockdown of ACTBL2 respectively NFAT5 on a protein level. Each siRNA knockdown was repeated and thus validated three times.

Western Blot

For basal expression analysis of ACTBL2, untreated adherent UWB1.289 cells were lysed for 15 minutes at 4°C using 300µl RIPA buffer (Sigma Aldrich Co., St. Louis, MO, USA) containing a previously 1:100 diluted protease inhibitor (Sigma Aldrich Co., St. Louis, MO, USA). After adding 100µl of 4x Laemmli sample buffer, the protein samples were loaded and separated according to their molecular weight using a 10% sodium dodecyl sulphate-polyacrylamide gel (Mini-PROTEAN TGX Precast Gels, Bio-Rad Laboratories Inc., Hercules, CA, USA) at a voltage of 70 V for 2 hours. After transferring the proteins onto a polyvinylidene fluoride membrane (Sequi-Blot PVDF Membrane, Bio-Rad Laboratories Inc., Hercules, CA, USA) for 65 minutes at 145mV and 4°C, the membrane was blocked for 1 hour at RT in 5% milk powder solution to prevent an unspecific antibody reaction. Subsequently, the membrane was incubated gently shaking overnight at RT with the following diluted primary antibodies: anti-ACTBL2 (1:500 dilution; rabbit IgG, polyclonal, abcam, ab100869) and ß-actin (1:1000 dilution; mouse IgG, monoclonal, Sigma, St. Louis, USA) with ß-actin serving as a control. Afterwards, the membranes were washed three times with TBS/Tween and subjected to the corresponding species-specific secondary antibodies (goat-anti-rabbit/mouse, 1:1000 dilution, Jackson Immuno Research, UK) for 1 hour at RT. After repeating the previously described washing steps, the antibody complexes were visualized using 5-bromo-4-chloro-3-indolylephosphate/nitro-blue-tetrazolium chloride (BCIP/NBT, Promega) in 0.1M Tris-HCl and 0.15M NaCl for 5-10 minutes. Western blotting analysis was performed using the Bio-Rad Universal Hood II and the corresponding software (Quantity One; Bio-Rad Laboratories Inc., Hercules, CA, USA). Each Western blot was repeated three times.

Cell Viability Assay and Proliferation Assay

For cell viability measurements a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, M-5655, 500 mg) colorimetric assay was conducted, while changes in cell proliferation were detected by performing a 5-bromo-2-deoxyuridine (BrdU) assay (Roche Cell Proliferation Elisa, BRDU (Colorimetric), Roche, Basel, Switzerland). In each assay executed, 5×103 UWB1.289 cells/100µl were seeded on sterile 96-well plates and maintained in culture overnight using RPMI 1640 GlutaMAX medium with 10% FBS. Subsequently, gene silencing of ACTBL2 respectively NFAT5 was performed as previously described. After 72h, both MTT and BrdU assay were conducted according to the manufacturer's protocol. An Elx800 universal Microplate Reader (BioTek, Winooski, VT, USA) was used to measure the optical density (OD) in each well at 595nm (MTT) and 450nm (BrdU). Each experiment was repeated and thus validated three times.

Wound Healing Assay

To analyze the cellular migration after gene silencing, 8×10^5 UWB1.289 cells/well were seeded on sterile 6-well plates and maintained in culture as previously outlined. After 24h, a sterile 200µl pipet tip was used to scratch a vertical line centrally into the monolayer, aiming to create an artificial wound. After gently washing the cells with PBS to remove excess cells, siRNA knockdown of each *ACTBL2* and *NFAT5* was performed as described above. To consequently monitor the cellular migration, digital images of each scratch were taken exactly 0h, 24h and 48h after the transfection by using an inverse phase-contrast microscope (Leica Dmi1, Leica, Wetzlar, Germany) and the according camera (Leica MC120 HD, Leica, Wetzlar, Germany). The images were subsequently analyzed by measuring the wounded areas at each time using ImageJ (https://imagej.nih.gov/ij/). The relative cell migration was outlined by calculating the difference of the covered area at 24h and 0h as well as 48h and 0h, and comparing the results to the untreated control.

RESULTS

ACTBL2 Expression in Epithelial Ovarian Cancer Correlates With Clinical and Pathological Characteristics

To examine the role of ACTBL2 in epithelial ovarian cancer, ACTBL2 expression was investigated in 156 specimens. Immunohistochemical staining of ACTBL2 was assessed in 134 cases (86%) in the cytoplasm and the cell membrane with a median (range) IRS of 4 (0,12) and 2 (0,8), respectively (**Tables S3A, B**). Positive ACTBL2 expression was defined and further investigated as combined cytoplasmic (IRS>2; n=117) and membranous (IRS>0; n=110) expression in the present cohort *via* ROC-curve analyses.

Consecutively performed correlation analyses of combined ACTBL2 expression and clinicopathological data revealed a significant positive correlation between high ACTBL2 expression and serous histology (**Table 2**; p=0.013, Cc=0.213). Moreover, high levels of ACTBL2 correlated significantly with high grading of serous carcinoma (**Table 2**; p=0.003, Cc=0.253).

Positive ACTBL2 Expression Is Associated With Impaired Overall Survival of EOC Patients

Intending to further investigate the prognostic significance of ACTBL2 expression in ovarian cancer, a univariate analysis of overall survival (OS) was performed.

In the present cohort, the patients' median age was 58.7 (± 31.4) years with a range of 31-88 years, while their median OS was 34.4 (± 57.8) months.

Combined cytoplasmic and membranous, thus positive ACTBL2 expression in EOC patients (n=101) was associated with a significantly shorter overall survival compared to patients with negative ACTBL2 expression (n=32; median OS 35.2 *vs.* 83.4 months; p=0.035) (**Figures 1A–F**).

TABLE 2 | Correlation analysis of ACTBL2 expression and clinicopathological data.

Variables	Combined ACTBL2 expression	
	p	Correlation coefficient
Histology		
serous	0.013*	0.213
clear cell	0.044*	-0.174
endometrioid	0.176	-0.118
mucinous	0.640	-0.041
FIGO	0.728	0.031
pT	0.150	0.126
pN	0.883	0.016
Grading		
serous – low grading	0.098	-0.144
serous – high grading	0.003*	0.253
clear cell, endometrioid and mucinous – G1 to G3	0.589	0.096

*Spearman's correlation analysis of combined cytoplasmic (IRS>2) and membranous (IRS>0) ACTBL2 expression and clinicopathological characteristics, showing a positive correlation between positive ACTBL2 expression, serous histology (p=0.013, Cc=0.213) and high grading of serous carcinoma (p=0.003, Cc=0.253), respectively. Significant correlations are indicated with asterisks (*p < 0.05).*
(p=two-tailed significance, Cc=correlation coefficient).

Positive ACTBL2 Expression and Clinicopathological Parameters Are Independent Prognostic Factors for Overall Survival

Aiming to detect which parameters are independent factors for overall survival in the present cohort, a multivariate Cox regression analysis was performed (**Table 3**). Patients' age (≤*60 vs.* >*60* years; p=0.011) as well as FIGO stage (FIGO I, II *vs.* III, IV; p<0.001) were confirmed as independent prognostic factors. Additionally, positive ACTBL2 expression (p=0.013), as previously defined, was found to be a novel and statistically independent prognostic factor for impaired overall survival of ovarian cancer patients. In contrast, tumor histology and nodal status were not independent in the established model.

ACTBL2 Expression Is Significantly Elevated in Ovarian Cancer Cell Lines, Showing Highest Level in Serous UWB1.289 Cells

The basal mRNA expression of *ACTBL2* was analyzed by qPCR in four EOC cell lines as well as in the benign cell line HOSEpiC (**Figure 2A**). All malignant cell lines displayed a significantly elevated *ACTBL2* expression compared to the benign control (p=0.028). Supporting our aforementioned results in immunohistochemistry, both serous cell lines OVCAR3 and UWB1.289 showed higher levels of *ACTBL2* than tested tumor cells of other histological subtypes. The BRCA1 mutant cell line UWB1.289 showed the comparatively highest ACTBL2 expression on mRNA as well as on protein level, whereas OVCAR3 cells showed a protein expression of ACTBL2 comparable to the non-serous cell lines used in this study (**Figure 2B**). Additionally executed immunocytochemical staining of UWB1.289 cells confirmed the cytoplasm and the cell membrane as locations of ACTBL2 expression, corroborating our findings from previous immunohistochemical analyses (**Figure 2C**).

Downregulation of *ACTBL2 In Vitro* Decreases Viability, Proliferation and Migration of UWB1.289 Cells, Indicating Its Functional Role in Serous Ovarian Cancer

Intending to elucidate the cellular function of ACTBL2 in terms of ovarian cancer etiology and progression, further *in vitro* experiments were performed. Since UWB1.289 cells showed the highest level of *ACTBL2*, this cell line was selected for additional investigations upon targeted gene silencing.

After proving the successful downregulation of ACTBL2 by both qPCR and immunocytochemistry (**Figure S2**), functional assays were executed to assess its impact on tumor cell biology. Given our previously described findings, we hypothesized that ACTBL2 might enhance cellular viability, proliferation and migration in ovarian cancer, thus serving as a potential explanation for the poor prognosis associated with positive ACTBL2 expression in EOC patients.

In each assay performed, the results obtained in UWB1.289 cells after siRNA knockdown of *ACTBL2* were compared to the results of an untreated control. As shown in **Figure 3**, successful downregulation of ACTBL2 led to a significant decrease in cellular viability (**Figure 3A**; p=0.008). Moreover, *ACTBL2* silencing significantly inhibited the proliferation of ovarian cancer cells (**Figure 3B**; p=0.012). In addition, siRNA transfected cells displayed a significantly reduced migration as compared to the untreated group (**Figures 3C–I**; p=0.012).

Summarizing, our findings suggest that the downregulation of ACTBL2 results in a significant decrease in viability, proliferation and migration of ovarian cancer cells, inversely supporting our hypothesis regarding the cellular function of ACTBL2.

Downregulation of *NFAT5 In Vitro* Regulates *ACTBL2* Expression and Consecutively Reduces Viability, Proliferation and Migration of UWB1.289 Cells

Aiming to assess molecular biological mechanisms regulating the function of ACTBL2, the impact of NFAT5 on ovarian cancer cells was further investigated.

Firstly, the basal mRNA expression of *NFAT5* was analyzed by qPCR accordingly to our aforementioned experiment regarding the basal expression of *ACTBL2* (**Figure 4A**). Again, all malignant cell lines used in our study showed a significantly elevated *NFAT5* expression compared to the benign control cell line HOSEpiC (*p=0.028, #p=0.027). Reflecting our previously revealed results concerning the mRNA expression of *ACTBL2*, UWB1.289 cells showed the highest level of *NFAT5*. Supporting the assumption of NFAT5 functioning as a transcription factor, immunocytochemical staining of UWB1.289 cells confirmed both cytoplasm and nucleus as locations of NFAT5 expression (**Figures 4B, C**).

In addition, *in vitro* experiments in UWB1.289 cells were performed to characterize the functional connection between ACTBL2 and its putative regulatory element NFAT5.

In a first step, *NFAT5* silencing was induced in the selected cell line by siRNA transfection. The successful downregulation of

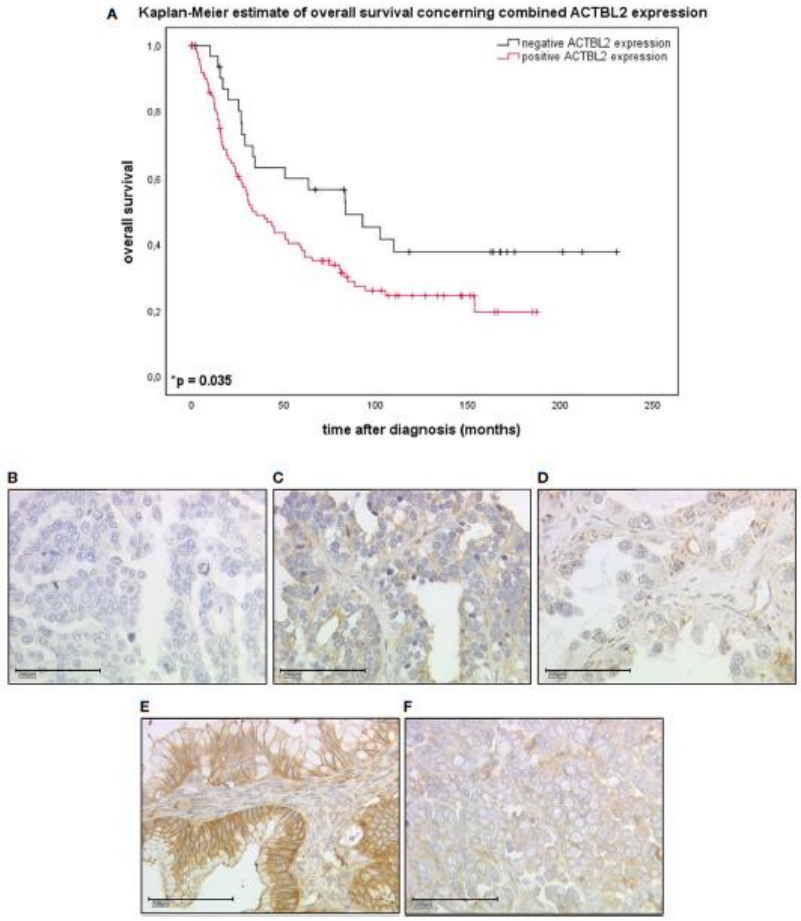

FIGURE 1 | Kaplan-Meier estimate of combined ACTBL2 expression in EOC patients as detected by immunohistochemistry. **(A)** Kaplan-Meier estimate (log-rank testing) concerning combined cytoplasmic (IRS>2) and membranous (IRS>0) ACTBL2 expression in epithelial ovarian cancer, being associated with impaired overall survival (median OS 35.2 *vs.* 83.4 months; p=0.035). Censoring events were marked in the graphs (+). **(B–F)** Detection of ACTBL2 by immunohistochemistry. Exemplary photographs (25x magnification; scale bar=100µm), showing the differences between ACTBL2-negative **(B)** and ACTBL2-positive tissue of all histological subtypes of EOC **(C–F)**, thus visually supporting the survival analysis displayed above: **(B)** serous carcinoma, cytoplasmic IRS=0, membranous IRS=0; **(C)** serous carcinoma, cytoplasmic IRS=4, membranous IRS=4; **(D)** clear cell carcinoma, cytoplasmic IRS=4, membranous IRS=1; **(E)** mucinous carcinoma, cytoplasmic IRS=8, membranous IRS=8; **(F)** endometrioid carcinoma, cytoplasmic IRS=8, membranous IRS=4.

NFAT5 on mRNA and protein level was proved by qPCR and immunocytochemistry, respectively (**Figure S3**). Moreover, the expression of *ACTBL2* after effectively performed *NFAT5* silencing was investigated by qPCR, showing a significant decrease of 46% after 48 hours (**Figure 5A**; p=0.008). Thus, we presumed that the downregulation of *ACTBL2* caused by *NFAT5* silencing would further lead to a decrease in cellular viability, proliferation and migration, reflecting our previously outlined results after *ACTBL2* knockdown. Consequently, we again performed the functional assays mentioned above, comparing the results obtained after siRNA knockdown of *NFAT5* to an untreated control. As shown in **Figure 5**, successful *NFAT5* silencing caused a significant decrease in cellular viability (**Figure 5B**; p=0.012) as well as significantly reduced cell proliferation rates (**Figure 5C**; p=0.001). Further, the downregulation of NFAT5, and consecutively *ACTBL2*, significantly inhibited the migration of UWB1.289 cells compared to the untreated control (**Figures 5D–J**; p=0.012).

TABLE 3 | Multivariate analysis.

Covariate	Hazard Ratio	95% CI	p-value
Patients' age (*≤60 vs. >60*)	1.830	1.151-2.910	0.011*
Histology	0.980	0.726-1.321	0.892
FIGO (*I, II vs. III, IV*)	4.295	2.004-9.206	<0.001**
Nodal status (*pNX/0 vs. pN1*)	0,935	0.578-1.514	0.785
positive ACTBL2 expression	2.034	1.161-3.564	0.013*

*Multivariate Cox regression analysis of ovarian cancer patients (n=156) and their clinicopathological characteristics considered in this study. Significant independent factors for overall survival in the present cohort are indicated with asterisks (*p < 0.05; **p < 0.001).*

In sum, our results show for the first time a functional relation between NFAT5 and ACTBL2 in ovarian cancer, with *NFAT5* silencing regulating the effect of ACTBL2 on cellular functions, predominantly resulting in a decreased migratory potential of UWB1.289 cells.

Cytoplasmic NFAT5 Expression in Epithelial Ovarian Cancer Correlates With Prognostically Favorable Clinical and Pathological Characteristics

In order to evaluate its impact in a clinical relation, NFAT5 expression was investigated in the previously described patient cohort (n=156, **Table 1**). NFAT5 staining was assessed in 127 cases (81%) in the cytoplasm (**Figure S4**) with a median (range) IRS of 0 (0,8) (**Table S3C**), while nuclear expression was only detected in 2 cases. Hence, considering its function as a transcription factor, NFAT5 was mainly present in its inactive form in the analyzed collective.

Additionally performed correlation analyses revealed significant correlations between cytoplasmic NFAT5 expression and clinicopathological characteristics (**Table S4**). Based on the thus detected results, Kruskal-Wallis-H tests were executed to further elucidate potential differences within FIGO stages and grading of serous carcinoma (**Figure 6**). Accordingly, low FIGO stages displayed a significantly higher cytoplasmic NFAT5 expression than advanced FIGO stages (**Figure 6A**; p=0.022). In addition, elevated cytoplasmic NFAT5 expression was significantly associated with low grading of serous carcinoma (**Figures 6B–D**; p<0.001).

Concluding, the presence of NFAT5 in the cytoplasm as an inactive transcription factor was linked to prognostically favorable clinical and pathological characteristics of epithelial ovarian cancer.

High Gene Expression of *ACTBL2* and *NFAT5* in Large Independent EOC Cohorts Is Significantly Associated With Impaired Overall Survival

Aiming to validate the prognostic impact of *ACTBL2* and *NFAT5* on overall survival respecting a larger collective of EOC patients, the Kaplan-Meier Plotter database was used (35). For both genes respectively, patients were divided into high- and low-expression groups based on gene-specific cut-off values, before accordingly executing analyses concerning overall survival. The survival time of

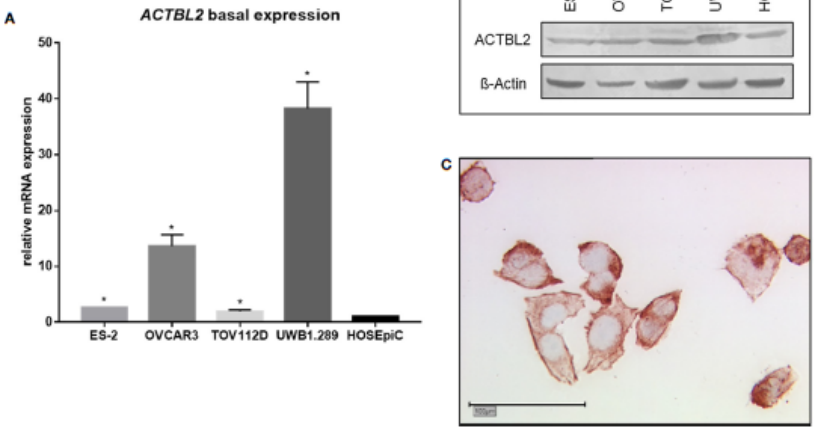

FIGURE 2 | Basal expression of ACTBL2 in ovarian cancer cell lines. **(A)** qPCR results showing *ACTBL2* expression in four EOC cell lines (ES-2, OVCAR3, TOV112D, UWB1.289) compared to the benign control cell line (HOSEpiC; n=6; *p=0.028). **(B)** Exemplary Western blot analysis of ACTBL2 expression (42 kD) in four EOC cell lines (ES-2, OVCAR3, TOV112D, UWB1.289) compared to the benign control cell line (HOSEpiC). **(C)** Detection of ACTBL2 in UWB1.289 cells by immunocytochemistry. Exemplary photographs (25x magnification; scale bar=100µm) showing protein expression in both cytoplasm and cell membrane.

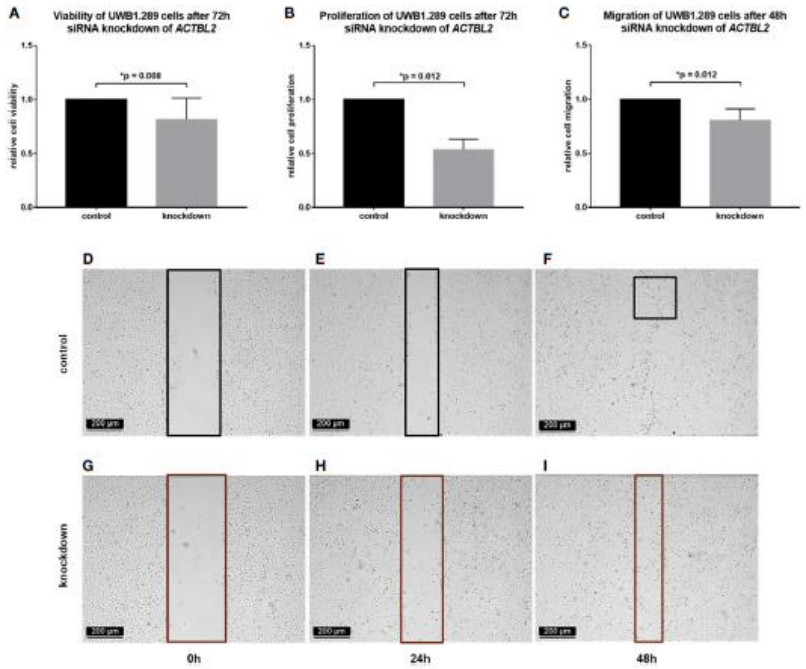

FIGURE 3 | Functional assays after *ACTBL2* silencing in UWB1.289 cells. **(A)** MTT assay results after siRNA (sequence 3) knockdown of *ACTBL2*, showing a significantly reduced cell viability (n=9, p=0.008). **(B)** BrdU assay results, displaying a significantly decreased cell proliferation after silencing of *ACTBL2* (n=8, p=0.012) **(C–I)** Wound healing assay results **(C)** after siRNA knockdown of *ACTBL2*, showing a significantly reduced migration of UWB1.289 cells after 48h **(G–I)** compared to the untreated control (**D–F**; n=8, p=0.012) (10x magnification; scale bar=200µm).

patients with high *ACTBL2* gene expression (n_{high}=272) was shown to be significantly shorter compared to patients of the low-expression group (n_{low}=101, p=0.036; **Figure S5A**), supporting our previously outlined results from immunohistochemical analyses regarding the prognostic relevance of cytoplasmic and membranous ACTBL2 expression in EOC patients.

Regarding *NFAT5* gene expression, comparable results were achieved by showing that high *NFAT5* gene expression (n_{high}=152) is significantly correlated with an impaired prognosis of ovarian cancer patients (n_{low}= 221; p=0.027; **Figure S5B**). Since the protein expression of NFAT5 was mainly detected in the cytoplasm of patients in our collective, being linked to prognostically favorable clinicopathological characteristics, a negative prognostic impact of nuclear NFAT5 expression can be assumed. Concordantly, although detected in very few cases in our cohort, nuclear protein expression of NFAT5 as a transcription factor of *ACTBL2* was associated with a significantly shorter overall survival of EOC patients (p=0.036; **Figure S5C**). However, as the survival analysis shown in **Figure S5B** is solely considering the gene expression of *NFAT5*, a more comprehensive analysis on the according protein distribution in each cellular compartment is required, to precisely allow a statement on its definite prognostic impact. An additionally performed correlation analysis of *ACTBL2* and *NFAT5* expression using the TIMER database (36) revealed a positive correlation trend between both genes (Cc=0.103, p=0.073; **Figure S5D**), hinting at their previously outlined functional relation in epithelial ovarian cancer.

DISCUSSION

In recent years, only very few studies have focused on Actin beta-like 2 and its molecular function. Whereas studies revealed an upregulation of ACTBL2 in pancreatic, colorectal and hepatocellular carcinoma, investigations focusing on its carcinogenetic impact in gynecological malignancies are still missing (16, 18, 19). Altered growth properties of hepatocellular carcinoma cells and a consecutively impaired disease-free survival of affected patients suggest a prognostic impact upon high intracellular protein abundance (16). By analyzing the expression pattern of Actin beta-like 2 in 156 EOC patients, we could show that ovarian cancer of high-grade serous histology displayed a significantly higher combined cytoplasmic and

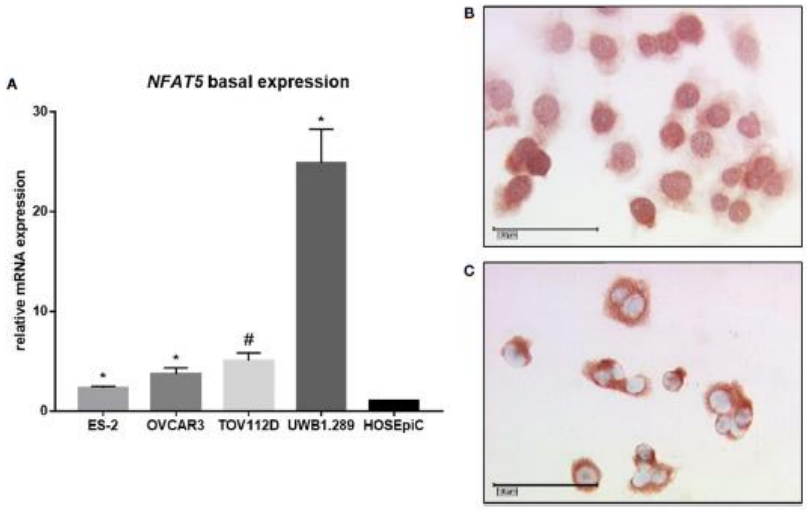

FIGURE 4 | Basal expression of NFAT5 in ovarian cancer cell lines. **(A)** qPCR results showing *NFAT5* expression in four EOC cell lines (ES-2, OVCAR3, TOV112D, UWB1.289) compared to the benign control cell line (HOSEpiC; n=6; *p=0.028; #p=0.027). **(B, C)** Detection of NFAT5 in UWB1.289 cells by immunocytochemistry. Exemplary photographs (25x magnification; scale bar=100µm) showing protein expression in both nucleus **(B)** and cytoplasm **(C)**.

membranous ACTBL2 expression than specimens of other histological subtypes. Consistently, the combined and thus positive ACTBL2 expression was associated with an impaired overall survival of affected patients and additionally being confirmed as a novel independent prognostic factor. In summary, our study provides for the first time significant evidence on the prognostic relevance of ACTBL2 expression in epithelial ovarian cancer.

Aiming at further elucidating the molecular function of Actin beta-like 2 regarding disease-promoting hence survival-limiting mechanisms, we focused on comprehensive *in vitro* analyses. Experiments assessing the basal expression of *ACTBL2* revealed significantly elevated *ACTBL2* levels in all tested ovarian cancer cell lines compared to the benign control. Consistent with our shown results regarding the expression in EOC patients, serous UWB1.289 cells showed the comparably highest ACTBL2 abundance on mRNA and protein level each. Providing knowledge on its molecular function, targeted gene silencing of *ACTBL2* in the selected cell line resulted in a reduced protein expression and a consecutively decreased cellular viability and migration.

Mazur et al. identified Actin beta-like 2 as a binding partner of gelsolin in human melanoma cells (20). Gelsolin, a multifunctional actin-binding protein, was shown to be present in the edge of lamellipodia and thus structures enriched in filamentous actin and involved in cellular migration (20, 37). A high expression of gelsolin in colorectal carcinoma was shown to increase the cellular migratory potential (38). As the proximity between gelsolin and polymerization competent ACTBL2 in lamellipodia was shown to be close enough for direct interaction, a congruent promigratory effect of Actin beta-like 2 was hypothesized (20, 21). As previously outlined, ACTBL2 expression was detected in the membrane of EOC cells by IHC as well as immunocytochemically *in vitro* in UWB1.289 cells. Since only combined cytoplasmic and membranous expression had a significant impact on patients' overall survival, the impaired prognosis might be based on the promigratory effect of Actin beta-like 2 provided by lamellipodia, being in line with pre-existing studies and supporting our results upon gene silencing of *ACTBL2*. Emphasizing the impaired OS of EOC patients upon positive ACTBL2 expression, a crucial favorable effect on metastatic processes can be assumed. Nonetheless, as the analyzed cohort contained very few cases of distant metastasis, more patients' data is yet to be collected to further assess the contribution of ACTBL2 to enhanced cellular motility in the course of ovarian cancer development with special regard to metastatic mechanisms.

Further, we observed a decline of 46% in cellular proliferation upon *ACTBL2* gene knockdown. While recent studies have only focused on ACTBL2 in a promigratory context (16, 21), our findings suggest an additional enhanced proliferative effect of Actin beta-like 2 in ovarian cancer cells, simultaneously underlining the observed correlation between high ACTBL2 expression in EOC patients and comparably fast proliferating serous carcinoma of high-grade histology. Intending to reveal putatively counteracting mechanisms on the function of ACTBL2, we assessed the regulatory impact of NFAT5 based on studies executed in vascular smooth muscle cells (21).

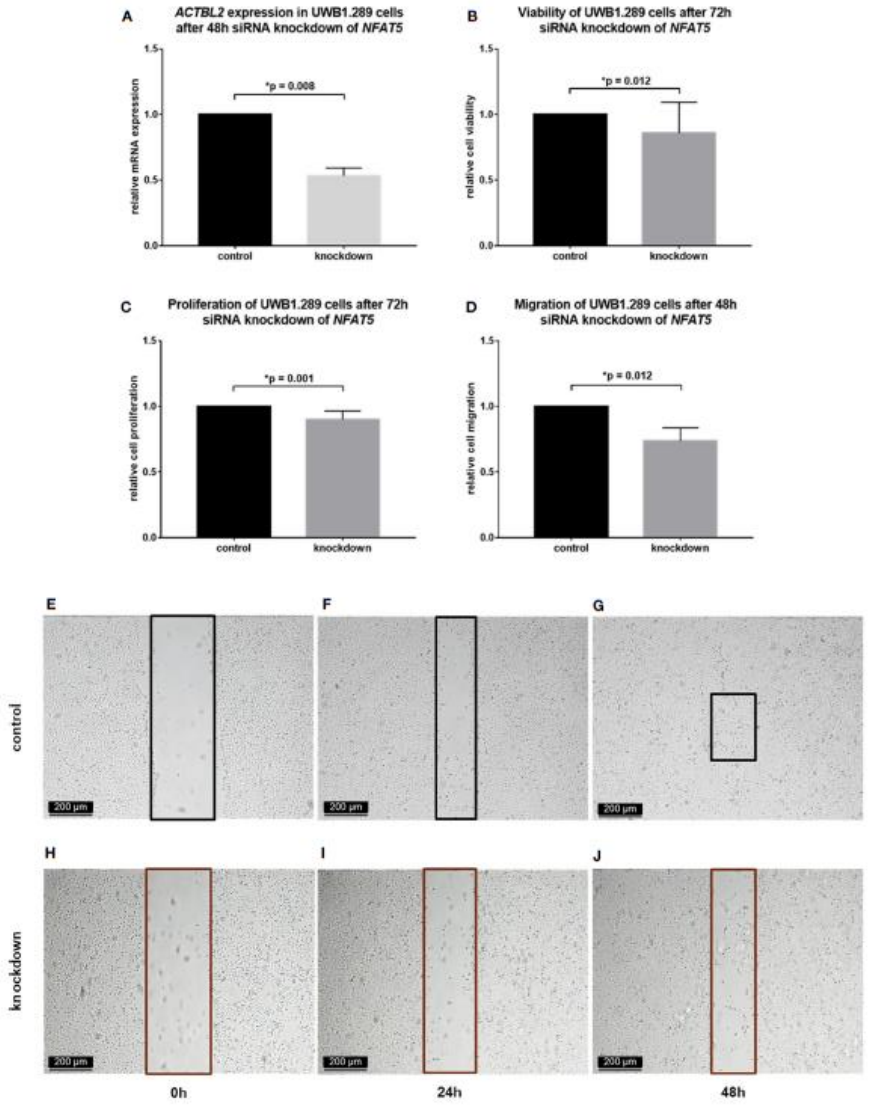

FIGURE 5 | Functional assays after *NFAT5* silencing in UWB1.289 cells. **(A)** *ACTBL2* expression in UWB1.290 cells after 48h siRNA (sequence 7) knockdown of *NFAT5*, proving its successful downregulation on mRNA level compared to the untreated control (p=0.008). **(B)** MTT assay results after siRNA knockdown of *NFAT5*, showing a significantly reduced cell viability (n=8, p=0.012). **(C)** BrdU assay results, displaying a significantly decreased cell proliferation after silencing of *NFAT5* (n=15, p=0.001) **(D–J)** Wound healing assay results **(D)** after siRNA knockdown of *NFAT5*, showing a significantly reduced migration of UWB1.289 cells after 48h **(H–J)** compared to the untreated control **(E–G**; n=8, p=0.012) (10x magnification; scale bar=200µm).

Hödebeck et al. showed that an siRNA induced gene knockdown of *NFAT5* resulted in a reduced cytoplasmic ACTBL2 expression of stretch stimulated VSMCs (21). NFAT5 itself is commonly known to be involved in enhancing cell migration and proliferation as well as to react to conditions of severe cellular osmotic stress (22–25). Nonetheless, the present study focused primarily on effects provided by NFAT5 upon ACTBL2 regulation. *In vitro* analyses of *NFAT5* in ovarian cancer

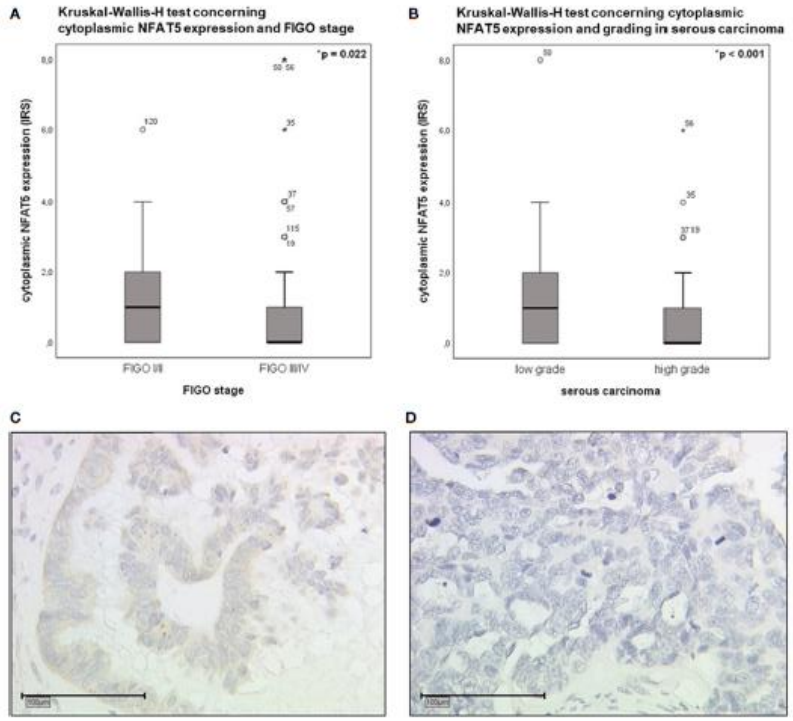

FIGURE 6 | Boxplot analysis of cytoplasmic NFAT5 expression (Kruskal-Wallis-H test). **(A)** Boxplot analysis of cytoplasmic NFAT5 expression comparing FIGO I,II (n=37, median IRS=1) to FIGO III,IV (n=89, median IRS=0; p=0.022). **(B)** Boxplot analysis of cytoplasmic NFAT5 expression in serous carcinoma (n=89) comparing low grading (n=26, median IRS=1) to high grading (n=63, median IRS=0; p<0.001). **(C, D)** Exemplary photographs (25x magnification; scale bar=100µm) of cytoplasmic NFAT5 expression in serous histological subtype, comparing positive expression in low grade (**C**, IRS=4) to negative expression in high grade (**D**, IRS=0) carcinoma.

revealed a significantly elevated mRNA expression in UWB1.289 cells, again being highest compared to other tested malignant cell lines. Protein expression of NFAT5 was detected in both nucleus and cytoplasm, reflecting its previously described function as a transcription factor of *ACTBL2* (21). Accordingly, downregulation of *ACTBL2* on mRNA level was successfully achieved by gene silencing of *NFAT5*. As viability and proliferation of UWB1,289 cells were consecutively diminished, a functional relation between NFAT5 and ACTBL2 in ovarian cancer was revealed for the first time. Moreover, a crucial role of ACTBL2 in cellular motility was again confirmed, reflected by a significantly declined cellular migration of 24% upon targeted *NFAT5* silencing. Taking clinical aspects into account, the presence of NFAT5 as an inactive transcription factor in EOC patients was linked to prognostically favorable characteristics, as a high cytoplasmic protein abundance correlated significantly with low FIGO stages and low grading of serous carcinoma.

Several studies provided evidence that nuclear translocation and activity of NFAT5 depend on posttranslational palmitoylation processes and are thus linked to cellular fatty acid oxidation (FAO) (39, 40). Targeted and irreversible inhibition of mitochondrial carnitine palmitoyltransferase 1 (CPT1) by Etomoxir resulted in a consecutively reduced cytoplasmic ACTBL2 abundance (21), since palmitoylation of NFAT5 was required to assure a nuclear entry within stretch-stimulated vascular smooth muscle cells (40). Apart from studies focusing on VSMCs, there is yet no evidence on the regulatory impact of Etomoxir on NFAT5 and ACTBL2 in cancer cells. The influence of FAO on carcinogenetic processes and consequently altered cellular functions upon irreversible CPT1 inhibition has been recently investigated in several tumor entities, demonstrating that Etomoxir might display a highly interesting and considerable therapeutic concept due to its antiproliferative effect (41–44). Nonetheless, Etomoxir was shown to simultaneously induce severe cellular oxidative stress *in vitro* (45) and *in vivo*, since a double-blind randomized phase II clinical trial on its therapeutic effect on congestive heart failure was prematurely stopped due to newly occurred hepatotoxicity (46). As our results confirmed a significant decrease in proliferation and migration of ovarian cancer cells upon specific downregulation of NFAT5 and ACTBL2, the

irreversible blockade of CPT1 provided by Etomoxir might display a new and more precise antiproliferative approach in oncology, assumptively diminishing the therapy-limiting cytotoxicity upon systemic treatment. Since ACTBL2 expression was shown to be associated with an impaired prognosis of ovarian cancer patients, putatively enhanced by its promigratory characteristics, a reduction of intracellular levels of ACTBL2 might result in prognostically favorable alterations in tumor biology. However, further experiments are required to assess the potential of Etomoxir of being a new putative mechanism to directly counteract the effects of increased ACTBL2 expression in ovarian cancer cells.

Concluding, the present study investigated the carcinogenetic and prognostic impact of ACTBL2 and NFAT5 in epithelial ovarian cancer by elucidating their expression pattern in EOC patients and their functional molecular interplay *in vitro*. Our results suggest ACTBL2 and its regulatory element NFAT5 to be of significant functional and prognostic importance in ovarian carcinogenesis by modulating cellular proliferation and motility. Further studies evaluating the targeted antiproliferative use of Etomoxir are necessary to precisely analyze its impact on NFAT5 and ACTBL2 expression *in vitro* and *in vivo* with special regard to consecutively altered cellular functions in epithelial ovarian cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Ludwig-Maximilians-University (LMU), Munich, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, UJ, BC, and NT. Validation, DM, CS, AB, SM, and FT. Formal analysis, NT, UJ, and BC. Investigation, NT, UJ, and BC. Writing - original draft preparation, NT and BC. Writing - review and editing, DM, CS, AC-R, SB, AH, FK, MZ, TKa, TKo, AB, SM, UJ, and FT. Visualization, NT. Supervision, DM, SM, UJ, and FT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.713026/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Characterization and prognostic impact of ACTBL2-positive tumor-infiltrating leukocytes in epithelial ovarian cancer.

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Abstract:

Actin beta-like 2 (ACTBL2) was recently identified as a new mediator of migration in ovarian cancer cells. Yet, its impact on tumor-infiltrating and thus migrating leukocytes (TILs) remains to date unknown. This study characterizes the subset of ACTBL2-expressing TILs in epithelial ovarian cancer (EOC) and elucidates their prognostic influence on the overall survival of EOC patients with special regard to different histological subtypes. Comprehensive immunohistochemical analyses of Tissue-Microarrays of 156 ovarian cancer patients revealed, that a tumor infiltration by ACTBL2-positive leukocytes was significantly associated with an improved overall survival (OS) (61.2 vs. 34.4 months; $p = 0.006$) and was identified as an independent prognostic factor (HR = 0.556; $p = 0.038$). This significant survival benefit was particularly evident in patients with low-grade serous carcinoma (OS: median not reached vs. 15.6 months, $p < 0.001$; HR = 0.058, $p = 0.018$). In the present cohort, ACTBL2-positive TILs were mainly composed of CD44-positive cytotoxic T-cells (CD8+) and macrophages (CD68+), as depicted by double-immunofluorescence and various immunohistochemical serial staining. Our results provide significant evidence of the prognostic impact and cellular composition of ACTBL2-expressing TILs in EOC. Complementary studies are required to analyze the underlying molecular mechanisms of ACTBL2 as a marker for activated migrating leukocytes and to further characterize its immunological impact on ovarian carcinogenesis.

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Characterization and prognostic impact of ACTBL2-positive tumor-infiltrating leukocytes in epithelial ovarian cancer

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Actin beta-like 2 (ACTBL2) was recently identified as a new mediator of migration in ovarian cancer cells. Yet, its impact on tumor-infiltrating and thus migrating leukocytes (TILs) remains to date unknown. This study characterizes the subset of ACTBL2-expressing TILs in epithelial ovarian cancer (EOC) and elucidates their prognostic influence on the overall survival of EOC patients with special regard to different histological subtypes. Comprehensive immunohistochemical analyses of Tissue-Microarrays of 156 ovarian cancer patients revealed, that a tumor infiltration by ACTBL2-positive leukocytes was significantly associated with an improved overall survival (OS) (61.2 vs. 34.4 months; $p = 0.006$) and was identified as an independent prognostic factor (HR = 0.556; $p = 0.038$). This significant survival benefit was particularly evident in patients with low-grade serous carcinoma (OS: median not reached vs. 15.6 months, $p < 0.001$; HR = 0.058, $p = 0.018$). In the present cohort, ACTBL2-positive TILs were mainly composed of CD44-positive cytotoxic T-cells (CD8+) and macrophages (CD68+), as depicted by double-immunofluorescence and various immunohistochemical serial staining. Our results provide significant evidence of the prognostic impact and cellular composition of ACTBL2-expressing TILs in EOC. Complementary studies are required to analyze the underlying molecular mechanisms of ACTBL2 as a marker for activated migrating leukocytes and to further characterize its immunological impact on ovarian carcinogenesis.

Abbreviations

ACTBL2	Actin beta-like 2
APC	Antigen-presenting cell
BRCA1/2	BReast CAncer 1/2
CI	Confidence interval
Cc	Correlation coefficient
CDK 4/6	Cycline-dependent kinase 4/6
cDNA	Complementary desoxy-ribonucleic acid
DAB	3,3′Diaminobenzidine
ECM	Extracellular matrix
EOC	Epithelial ovarian cancer
F-actin	Filamentous actin
FBS	Fetal bovine serum
FFPE	Formalin-fixed and paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
HGSC	High-grade serous carcinoma

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HR	Hazard Ratio
HRD	Homologous recombination deficiency
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
LGSC	Low-grade serous carcinoma
LMU	Ludwig-Maximilians-University
MAP-kinase	Mitogen-activated protein kinase
MCR	Munich Cancer Registry
mRNA	Messenger ribonucleic acid
NFAT5	Nuclear factor of activated T-cells 5
OS	Overall survival
PARPi	Poly-ADP-ribose-polymerase inhibitor
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT	Room temperature
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
TAM	Tumor-associated macrophage
TILs	Tumor-infiltrating leukocytes
TMA	Tissue-Microarray
TMB	Tumor mutational burden
VSMC	Vascular smooth muscle cell
WHO	World Health Organization

Epithelial ovarian cancer (EOC) remains the fifth leading cause of cancer death in women and the most lethal tumor entity among gynecological cancer patients[1]. As a result of insufficient screening methods and a comparably late onset of primarily unspecific symptoms, EOC is mostly detected in an advanced stage with a consequently poor five-year survival rate of 47%[2]. Apart from the FIGO stage at initial diagnosis, decisive prognostic factors for overall survival include patient's age, histological subtype, tumor grade and the volume of residual disease after primary debulking surgery as the most significant ones[3–6]. Although recent studies stated substantial differences in ovarian carcinogenesis regarding underlying molecular pathways and clinicopathological features[7–9], first-line therapy of EOC to date still consists of cytoreductive surgery followed by adjuvant platinum-based chemotherapy, irrespective of distinct histological subtypes[10]. Maintenance treatment contains the use of VEGF-inhibitor bevacizumab[11,12] and/or poly-ADP-ribose-polymerase inhibitors (PARPi), depending on the patient's prior response to chemotherapy and the individual BRCA1/2 mutation and homologous recombination deficiency (HRD) status[13,14]. In contrast to other gynecological malignancies such as cervical and endometrial carcinoma, ovarian cancer shows the least susceptibility to immune therapy due to its comparably low tumor mutational burden (TMB)[15–18]. Despite several attempts to establish checkpoint inhibitors as a promising new option in EOC treatment, no significant prognostic benefit could be shown thus far[19,20]. Further development of targeted therapeutical approaches requires a definition of predictive factors for the response to immunotherapy and an increasingly better understanding of the distinct tumor biology and microenvironment.

Actin beta-like 2 (ACTBL2) is considered a newly discovered non-muscle actin isoform with 92% structural similarity to ß-actin[21,22]. Yet, recent studies corroborated a genetic distance from the six commonly known isoforms, with *ACTBL2* showing the highest number of non-conserved amino acid substitutions in comparative phylogenetic analyses[23]. Functional examinations in human melanoma cells revealed an interaction between ACTBL2 and gelsolin in the course of cellular lamellipodia formation[24]. Subsequent studies emphasized its significant motility-enhancing effect since a lack of ACTBL2 was associated with impaired cellular invasion abilities and an altered actin cytoskeleton structure[23]. Consistent with that, gene silencing of *ACTBL2* and its transcription factor Nuclear factor of activated T-cells 5 (*NFAT5*) resulted in decreased migration of biomechanically activated vascular smooth muscle cells (VSMC)[25]. Besides a significant upregulation in colorectal and hepatocellular carcinoma, *ACTBL2* was additionally identified as a potential risk gene in ovarian cancer[26–28]. Comprehensive analyses confirmed a statistically independent prognostic disadvantage for EOC patients with impaired overall survival upon positive ACTBL2-expression in the according tumor cells[29]. Further, functional assays after targeted gene silencing proved its significant modulating impact on proliferation and especially migration of high-grade serous ovarian carcinoma cells[29]. Despite growing evidence on its crucial and fundamental impact on cellular motility, the extent to which ACTBL2 is expressed in tumor-infiltrating and thus migrating leukocytes (TILs) remains to date unknown.

The present study aims at identifying and characterizing the subset of ACTBL2-expressing TILs in epithelial ovarian cancer and at elucidating their prognostic influence on the overall survival of EOC patients with special regard to different histological subtypes.

Methods

Ethical approval

The present study was carried out according to the guidelines of the Ethics Committee of the Ludwig-Maximilians-University (LMU), Munich, Germany (approval number 227-09, 18-392 and 19-972). All tissue samples

utilized were derived from material, which was primarily used for histopathological assessment and stored in the archives of our Department of Obstetrics and Gynecology, LMU, Munich, Germany. Diagnostic procedures on the tumor tissue were completed before its scientific use, securing a full anonymization of the patients' data during all experimental and analytical stages. All experiments were performed in strict compliance with the standards of the Declaration of Helsinki (1975), given the written informed consent of all patients/participants.

Patients and specimens

Tissue samples of 156 patients who underwent cytoreductive surgery for EOC between 1990 and 2002 at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University in Munich were collected, with distinct biopsies of representative tumor areas being combined in a Tissue-Microarray (TMA) by the Department of Pathology, LMU, Munich. The corresponding clinical data was gained from the patients' charts and the consecutive follow-up data was provided by the Munich Cancer Registry (MCR). None of the patients has had neoadjuvant chemotherapy in the clinical course and only patients with pathologically confirmed EOC were included in the collective. Patients with benign, precursor or borderline lesions were accordingly excluded from the study.

All samples were formalin-fixed and paraffin-embedded (FFPE) prior to being assessed by specialized gynecological pathologists regarding histopathological criteria. The specimens were classified into the four most common histological subtypes [serous (n = 110), clear cell (n = 12), endometrioid (n = 21) and mucinous (n = 13) carcinoma; Table 1] and consecutively graded respecting the currently valid WHO classification[30]. Serous ovarian cancer tissue was subdivided into low and high grading. Samples of endometrioid histology were graded from G1 to G3 as well as tissue from mucinous carcinoma since this subtype is lacking explicit WHO classification criteria. Clear cell cancer was always categorized as G3. Tumor staging was executed in line with the FIGO classification [I (n = 35), II (n = 10), III (n = 103), IV (n = 3)] based on available data on primary tumor extension (n = 155) according to the TNM classification as a globally recognized standard for the primary tumor site and size (T), regional lymph node involvement (N) and the presence of distant metastases (M)[31]. Regarding lymph node involvement, data was accessible in 95 cases [N0 (n = 43), N1 (n = 52)], whereas data on distant metastasis by the time of cytoreductive surgery was only obtainable in 9 cases [M0 (n = 3), M1 (n = 6)]. Information on the FIGO stage and histological grading are unavailable in 5 and 12 cases, respectively.

Serial tissue slides

For a distinct characterization of particular cells by comparative immunohistochemical analyses, serial slides of selected patients' tissue of each histological subtype from the previously described collective were produced. Formalin-fixed and paraffin-embedded (FFPE) ovarian cancer tissue was cut into at least four successive, 3 µm thick slices using a sledge-microtome (Hn 40, Reichert-Jung, Germany). After stretching the slices in a water bath, the tissue was placed on ascending numbered microscope slides (Menzel-Gläser Superfrost Plus, ThermoScientific, Gerhard Menzel GmbH, Braunschweig) and was dried for 12 h in an incubator at 50 °C. Serial tissue slides from primary fallopian tube cancer were provided by the Department of Pathology, LMU, Munich.

Immunohistochemistry

Immunohistochemical staining was conducted as previously described[29]. Formalin-fixed and paraffin-embedded tissue slides were dewaxed in Roticlear (Roth, Karlsruhe, Germany) for 20 min and then shortly washed in 100% ethanol. After blocking the endogenous peroxidase by using 3% H_2O_2 in methanol for 20 min, the samples were gradually rehydrated in descending ethanol concentrations (100%, 70% and 50%) before being put in distilled water. In a next step, the slides were placed in a pressure cooker containing a boiling sodium citrate buffer (0.1 M citric acid, 0.1 M sodium citrate; pH = 6) and were followingly heated for 5 min. After cooling, the specimens were washed again in distilled water and afterwards twice in phosphate-buffered saline (PBS) for 2 min each. Intending to avoid an unspecific staining reaction, a blocking solution [Reagent 1; ZytoChem Plus HRP Polymer System (mouse/rabbit); Zytomed, Berlin, Germany] was applied on the tissue for 5 min at room temperature (RT) prior to the incubation with distinct primary antibodies under reagent-specific conditions as listed in detail in the Supplementary file, Table S1. Following that, the slides were washed twice in PBS and subsequently treated with a post-block solution [Reagent 2; ZytoChem Plus HRP Polymer System (mouse/rabbit); Zytomed, Berlin, Germany] for 20 min at RT before another 30 min incubation with an HRP-polymer, containing bound anti-mouse and anti-rabbit antibodies [Reagent 3; ZytoChem Plus HRP Polymer System (mouse/rabbit); Zytomed, Berlin, Germany]. The staining was visualized by applying 3,3′diaminobenzidine (DAB) and the according substrate buffer (Liquid DAB and Substrate Chromogen System; DAKO, Munich, Germany) on the tissue. After washing the slides in distilled water to end the reaction, Mayer's acidic hemalum (Waldeck, Münster, Germany) was used for counterstaining. Next, the tissue was dehydrated in a series of ethanol with rising concentrations (70%, 96% and 100%) before being put in Roticlear and subsequently being covered. Kidney, placenta, colon and tonsil tissue served as negative and positive controls to determine the most suitable antibody dilution and to prove the specificity of the immunoreaction (Figure S1). Regarding the negative controls, each primary antibody was replaced by a species-specific isotype control antibody (BioGenex, Fremont, CA, USA).

Immunofluorescence

For immunofluorescence staining, the FFPE slides were pre-treated as previously described for immunohistochemistry. In order to prevent an unspecific binding of the primary antibodies, a blocking solution (Ultra Vision Protein Block; ThermoScientific, Lab Vision, Fremont, CA, USA) was applied on the tissue for 15 min at RT. After gently removing the surplus of blocking solution, the slides were incubated with a mixed solution of primary antibodies against ACTBL2 and CD45, respectively CD44, for 16 h at 4 °C (for detailed information

Clinicopathological parameters	n	Percentage (%)
Histology		
Serous	110	70.5
Clear cell	12	7.7
Endometrioid	21	13.5
Mucinous	13	8.3
Primary tumor extension		
TX	1	0.6
T1	40	25.6
T2	18	11.5
T3	97	62.3
Nodal status		
pNX	61	39.1
pN0	43	27.6
pN1	52	33.3
Distant metastasis		
pMX	147	94.2
pM0	3	1.9
pM1	6	3.8
Grading serous		
Low	24	21.8
High	80	72.7
Grading endometrioid		
G1	6	28.6
G2	5	23.8
G3	8	38.1
Grading mucinous		
G1	6	46.2
G2	6	46.2
G3	0	0
Grading clear cell		
G3	12	100.0
FIGO stage		
I	35	22.4
II	10	6.4
III	103	66.0
IV	3	1.9
Patients' age		
≤ 60 years	83	53.2
>60 years	73	46.8

Table 1. Clinicopathological features of 156 ovarian cancer patients included in this study.

on all antibodies used, see Supplementary file, Table S1). Next, the slides were washed twice in PBS and treated with fluorophore-labelled and species-specific secondary antibodies (Table S2) in the dark for 30 min at RT. Following, the specimens were once again washed twice in PBS prior to being covered in a dry state with mounting medium containing DAPI for nuclear counterstaining [Vectashield Anti-fade mounting medium with DAPI (H-1200); Vector Laboratories, Burlingame, CA, USA]. The double-staining was observed by using a confocal laser microscope (Axiophot fluorescence microscope; Zeiss, Oberkochen, Germany) and subsequently analyzed with the corresponding software AxioVision.

Antibody specificity and validation

For proving the specificity of the used anti-ACTBL2 antibody, in vitro experiments upon small interfering RNA (siRNA) knockdown of *ACTBL2* were performed as previously described[29]. UWB1.289 cells (serous ovarian cancer, BRCA1 negative—ATCC, Rockville, MD, USA) were seeded on sterile 6-well plates and maintained in culture using RPMI 1640 GlutaMAX Medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK) in a humified incubator at 37 °C and 5% CO_2 until reaching a cell density of 70%. Afterwards, transfection was executed by using siRNA for *ACTBL2* (GeneSolution siRNA, Qiagen Sciences, MD, USA; for detailed information on corresponding sequences, see Figure S2 g) and Lipofectamine RNAiMAX reagent

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol in OptiMEM Reduced Serum Medium (ThermoFisher Scientific, Waltham, MA, USA). After 48 h of incubation under the above mentioned conditions, the cells were harvested and used for mRNA isolation using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). 1 µg RNA was utilized for conversion into cDNA by utilizing the MMLV Reverse Transcriptase 1st-strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA). mRNA expression of *ACTBL2* after siRNA knockdown was quantified by qPCR using FastStart Essential DNA Probes Master and gene-specific primers (Roche, Basel, Switzerland, Figure S2 g). The relative expression was calculated for each sequence by the $2^{-\Delta\Delta Ct}$ formula[32] using *GAPDH* and *ß-actin* as housekeeping genes. Figure S2 a shows the successful and significant downregulation of *ACTBL2* up to 90% (sequence 3). Each siRNA knockdown and qPCR were repeated three times.

In order to prove a concordant decrease of ACTBL2 on a protein level, immunocytochemical (ICC) staining after 48 h of *ACTBL2* knockdown in UWB1.289 cells was executed. 5×10^4 UWB1.289 cells were seeded per well in sterile 4-well chamber slides (Lab-Tek II Chamber Slides, ThermoFisher Scientific, Denmark) and maintained in culture overnight before executing siRNA knockdown of *ACTBL2* as previously described. Next, the slides were washed twice for 5 min with PBS before being fixed in 100% methanol and ethanol (1:1) at room temperature for 15 min. After drying, the slides were treated with goat-derived serum (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) for 20 min at room temperature to avoid unspecific background staining. After another washing step, the slides were incubated with the aforementioned primary anti-ACTBL2 antibody (Table S1) in a 1:400 dilution for 16 h at 4 °C overnight. Following, a biotinylated secondary anti-rabbit antibody (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) was applied for 30 min before subsequently treating the slides with an avidin–biotin-peroxidase complex (Vectastain Elite rabbit-IgG-kit, Vector Laboratories, Burlingame, CA, USA) for another 30 min. The staining was visualized by using chromogen 3-amino-9-ethylcarbazole (AEC+, DAKO, Hamburg, Germany) for 10 min with the reaction being stopped by placing the slides in distilled water. Counterstaining was executed with Mayer's acidic hemalum (Waldeck, Münster, Germany) before finally covering the slides using an aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany). The results obtained are shown in Figure S2 b–f, displaying a significant decrease in intracellular ACTBL2 expression.

Staining evaluation and statistical analysis

Specimens of all 156 EOC patients were analyzed after the successfully performed immunohistochemical staining of ACTBL2 with special regard to the presence of ACTBL2-expressing TILs by using a Leitz photomicroscope (Wetzlar, Germany). Patients were divided binarily into two groups (0 = ACTBL2-positive TILs *not* detectable, 1 = ACTBL2-positive TILs detectable), thus enabling subsequent statistical analyses using IBM SPSS Statistics 28.0 (IBM Corporation, Armonk, NY, USA). Spearman's analysis[33] was executed to calculate bivariate correlations between pre-existing clinicopathological data and the current staining results. The overall survival was visualized by Kaplan–Meier estimates with the log-rank testing being used to check for statistical significance[34]. Appropriate cut-off values were primarily selected by performing a ROC curve analysis, being a reliable and widely recognized method for cut-off point definition[35]. Additionally, the Youden index was used to optimize the cut-off values by maximizing the sum of sensitivity and specificity[36,37]. For multivariate analyses, a Cox regression model considering clinicopathological characteristics and the investigated parameters was established[38]. For all analyses performed, p-values ≤ 0.05 were considered statistically significant.

Results

Tumor infiltration by ACTBL2-positive leukocytes is significantly associated with improved overall survival of ovarian cancer patients

Intending to investigate the distribution of ACTBL2-positive tumor-infiltrating leukocytes, immunohistochemical staining was analyzed in a cohort of 156 ovarian cancer patients. Tissue of 128 patients (82% of all evaluable cases) could be assessed regarding the specific presence of ACTBL2-expressing TILs, being binarily divided into two groups. In 99 out of 128 cases, an infiltration by ACTBL2-positive leukocytes was detected (77%), whereas in 29 cases (23%) such intra-tumoral leukocyte spreading could not be shown (Fig. 1c). Representative photographs of ACTBL2-expressing TILs in all considered histological subtypes are depicted in Fig. 1d–h, demonstrating that the staining intensity and thus cytoplasmic ACTBL2 expression in leukocytes was remarkably higher than the ACTBL2 level in tumor cells throughout all specimens.

Consecutively performed correlation analyses with clinicopathological data revealed a significant negative correlation between patients' age and the occurrence of ACTBL2-positive TILs (Table S3; $Cc = -0.226$, $p = 0.009$). In addition, an infiltration by ACTBL2-expressing leukocytes was significantly associated with low grading of serous carcinoma (Table S3; $Cc = 0.200$, $p = 0.025$).

Aiming to further delineate the prognostic significance of ACTBL2-expressing leukocytes in EOC, a univariate analysis regarding the overall survival (OS) was executed. The patients' median age in the present cohort (n = 156) was 58.7 (standard deviation (SD) = 31.4) years with a range from 20.7–88.0 years, while their median OS amounted 33.8 (SD = 57.8) months. A tumor infiltration by ACTBL2-positive leukocytes was found to be significantly associated with improved overall survival of EOC patients (Fig. 1a; 61.2 (n = 99) vs. 34.4 (n = 29) months; $p = 0.006$).

Considering the previously described significant correlation with low-grade serous histology, the impact of ACTBL2-expressing leukocytes on patients' OS in this distinct subgroup (n = 24) was further evaluated. The median age of the corresponding patients was 50.0 (SD = 13.4) years with a median survival time of 105.4 (SD = 63.6) months. Emphasizing the outlined results concerning the overall collective, a highly significant prognostic benefit of patients with detectable ACTBL2-positive TILs and low-grade serous carcinoma (LGSC) was revealed (Fig. 1b; median not reached (n = 17) vs. 15.6 months (n = 4); $p < 0.001$).

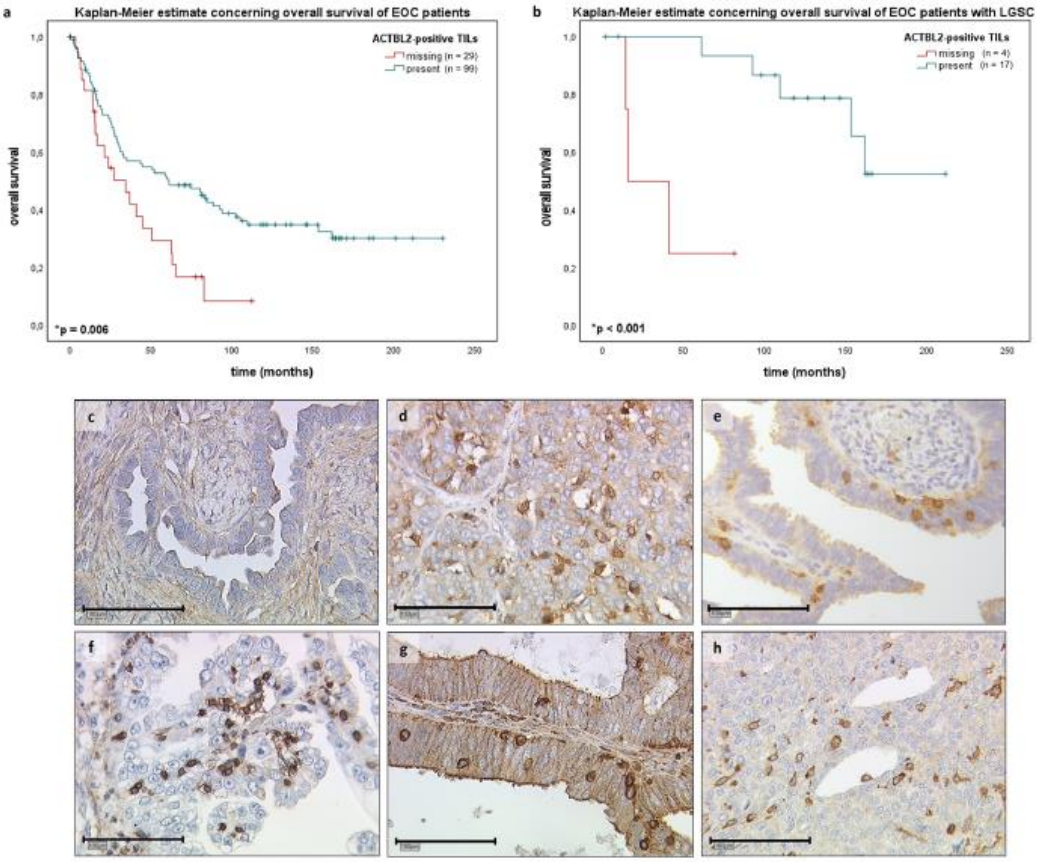

Figure 1. Kaplan–Meier estimates and exemplary photographs of tumor infiltration by ACTBL2-positive leukocytes as detected by immunohistochemistry. **(a)** Kaplan–Meier estimate (log-rank testing) considering the presence of ACTBL2-positive TILs in the overall collective of 156 EOC patients, being associated with a significantly longer overall survival (61.2 vs. 34.4 months; $p = 0.006$). **(b)** Kaplan–Meier estimate (log-rank testing) regarding the occurrence of ACTBL2-expressing TILs in low-grade serous ovarian cancer tissue, showing a significant survival benefit of the corresponding patients (median not reached vs. 15.6 months; $p < 0.001$). **(c)** Exemplary photograph of serous ovarian cancer without ACTBL2-positive tumor-infiltrating leukocytes. **(d–h)** Detection of ACTBL2-positive TILs by immunohistochemistry. Representative photographs of all considered histological subtypes in the given study. Throughout all specimens, the cytoplasmic ACTBL2 expression in leukocytes was remarkably higher than the ACTBL2 expression in tumor cells of **(d)** high-grade serous, **(e)** low-grade serous, **(f)** clear cell, **(g)** mucinous and **(h)** endometrioid ovarian carcinoma (x25 magnification, scale bar = 100µm).

The presence of ACTBL2-expressing TILs is an independent prognostic factor for overall survival

For the detection of independent prognostic factors for overall survival in the analyzed cohort, a multivariate Cox regression analysis was executed (Table 2). Histological grading (Hazard Ratio (HR) = 1.841, $p = 0.001$), as well as FIGO stage (HR = 2.099, $p < 0.001$), were confirmed as statistically independent factors in the overall collective. Moreover, the presence of ACTBL2-positive TILs in EOC patients' tissue, regardless of the histological subtype, was found to be a novel and independent prognostic factor for overall survival with a Hazard Ratio of 0.556 ($p = 0.038$). Since an infiltration by ACTBL2-expressing immune cells was shown to play a significant prognostic role especially in patients with LGSC, a separate Cox regression was calculated for this specific subgroup. In line with the obtained results concerning the total cohort, an infiltration by ACTBL2-positive leukocytes was identified as a statistically independent prognostic marker for the overall survival of patients with low-grade serous ovarian cancer (HR = 0.058, $p = 0.018$).

Covariate	Overall collective			Low-grade serous carcinoma		
	Hazard Ratio	95% CI	*p* value	Hazard Ratio	95% CI	*p* value
Patients' age (≤ 60 vs. > 60)	1.301	0.804–2.106	0.284	1.909	0.428–8.521	0.397
Histology	0.960	0.710–1.297	0.789	–	–	–
Grading	1.841	1.275–2.660	0.001*	–	–	–
FIGO stage	2.099	1.402–3.145	< 0.001**	1.600	0.557–4.597	0.383
ACTBL2-positive TILS	0.556	0.319–0.969	0.038*	0.058	0.005–0.618	0.018*

Table 2. Multivariate analysis. Multivariate Cox regression analysis regarding the overall collective (n = 156) and the subgroup of patients with low-grade serous carcinoma (LGSC; n = 24) and their clinicopathological features as considered in the present study. As the distinct cases of patients with LGSC have been pre-selected before executing the calculation, data on histology and grading are consequently not available. Significant independent prognostic factors for overall survival in this cohort are indicated with asterisks (*$p < 0.05$; **$p < 0.001$).

ACTBL2-positive TILs in EOC are mainly composed of CD44-positive cytotoxic T-cells and macrophages

Aiming at characterizing the subset of ACTBL2-expressing TILs in epithelial ovarian cancer with special regard to putative immune-mediated antitumoral effects, immunofluorescence double-staining was performed. In the first step, exemplary tissue slides of each histological subtype with previously detected leukocyte infiltration in the corresponding TMA were double-stained with antibodies against ACTBL2 and CD45 as a common leukocyte antigen to demonstrate the high protein expression of ACTBL2 in TILs in general (Fig. 2a–f). Assuming that ACTBL2 plays a significant role in cellular motility and migration of activated leukocytes, CD44 as a commonly known adhesion molecule and regulator of intra-tumoral leukocyte movement[39,40] was chosen for subsequent analyses. As depicted in Fig. 2g–l, ACTBL2-positive cells showed a clear co-expression of CD44. For an even more precise cellular characterization, serial tissue slides of the according specimens were produced, thus enabling a consecutive immunohistochemical staining series with up to four antibodies and an exact identification of singular cells in each histological subtype. As exemplarily shown in Fig. 3a–d for clear cell and Fig. 4a–d for serous carcinoma, tumor-infiltrating leukocytes showed a high intracellular level of ACTBL2 as well as a strong expression of membrane-bound CD44. Intending to define the exact cellular composition of ACTBL2-expressing leukocytes in ovarian cancer, various common markers for the most frequent immune cell subtypes have been investigated—CD4 (T-helper cells), FOXP3 (regulatory T-cells) and CD56 for natural killer cells. None of the mentioned markers could be detected in ACTBL2-positive TILs (data not shown). Instead, complementary serial staining identified CD8-expressing cytotoxic T-cells and CD68-positive macrophages as the predominant cellular subtypes (Figs. 3e–g, 4e–h). Further exemplary photographs and results of immunofluorescence double-staining as well as immunohistochemical serial staining of all remaining histological EOC subtypes are shown in Figures S3–S6.

Discussion

Recent studies provided growing evidence on the impact of Actin beta-like 2 on cellular motility by elucidating its molecular function in the course of lamellipodia formation. Comprehensive analyses of human melanoma cells revealed a specific interaction between polymerized ACTBL2 and the multifunctional actin-binding protein gelsolin in the edge of lamellipodia as protrusions conducting cellular migration[24,41]. Moreover, Malek et al. proved a significant change in the actin cytoskeleton structure upon knockout of *ACTBL2* resulting in impaired cellular invasion abilities and altered invadopodia and focal adhesion formation[23]. Despite the given findings, the extent to which ACTBL2 is expressed in tumor-infiltrating leukocytes as cells with high migratory potential and activity remains to date unknown.

The present study is the first one to ever investigate the expression of ACTBL2 in TILs with special regard to their prognostic significance on the overall survival of ovarian cancer patients. By examining the presence of ACTBL2-positive TILs in 156 EOC specimens via immunohistochemistry we could demonstrate that the expression of ACTBL2 in leukocytes was remarkably higher than its level in the according tumor cells. Subsequent survival analyses confirmed a significant and statistically independent prognostic benefit for patients with detectable ACTBL2-expressing TILs. Assuming that the composition of the given leukocyte subset is crucial for the favorable prognosis, further comprehensive staining series were executed to precisely define the distinct cellular subtypes in terms of immune-mediated antitumoral effects. Since effective leukocyte homing is indispensable for a successful tumor invasion, we consequently examined the co-expression of ACTBL2 and CD44 in TILs, presuming a significant interplay between cellular adhesion provided by CD44 and a consecutive promigratory rearrangement of the actin cytoskeleton.

Mrass et al. showed that CD44, a surface glycoprotein and adhesion receptor for extracellular matrix (ECM) proteins and glycosaminoglycans, is localized in small cellular cell protrusions at the rear end of crawling T-cells[39]. Whereas its extracellular domain is essential for a close interaction with ECM fibers by promoting cellular attachment, the intracellular domain was shown to contain several binding sites for signaling molecules, providing a linkage between the actin cytoskeleton and membrane proteins and thus regulating cell migration[39,42,43]. Consequently, CD44-deficient cytotoxic T cells showed a significantly reduced migratory potential, indicating its

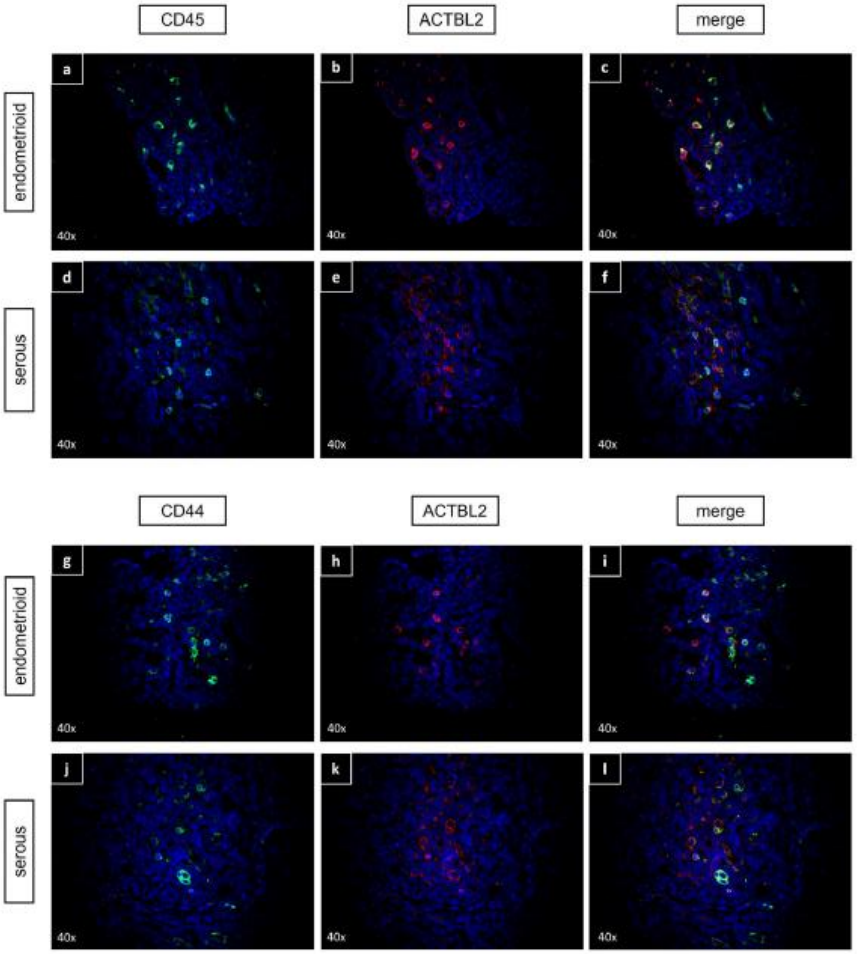

Figure 2. Immunofluorescence double-staining with anti-ACTBL2, anti-CD45 and anti-CD44 antibodies. (**a–f**) Representative staining results of EOC tissue of endometrioid (**a–c**) and serous (**d–f**) histology, showing a high ACTBL2 expression in CD45-positive tumor-infiltrating leukocytes. (**g–l**) Exemplary photographs of endometrioid (**g–i**) and serous (**j–l**) carcinoma, proving a co-expression of ACTBL2 and membrane-bound CD44 as a marker for activated TILs (×40 magnification).

substantial impact on cellular polarity and intra-tumoral navigation[39]. Focusing on cellular structures enhancing motility, CD44s as a specific splice isoform was identified as an integral element in invadopodia of tumor cells[44]. Invadopodia are dot-shaped and actin-rich protrusions with the ability to degrade the ECM, hence enabling the invasion of cancer cells[45]. Zhao et al. stated a significantly suppressed invadopodia activity and diminished invasiveness upon shRNA-mediated depletion of CD44s[44]. Podosomes, similar protrusive structures with an actin-rich core, are mainly located at the ventral site of e.g. vascular smooth muscle cells and antigen-presenting cells (APC) like dendritic cells (DC) and macrophages[45]. Culturing of the respective cells in collagen resulted in the formation of filamentous actin-rich protrusions containing accumulated podosome-associated proteins such as ß1-integrin and gelsolin as well as CD44, which was significantly associated with the proteolytic activity of human macrophages[45,46]. Consistent with the given evidence of the significant molecular impact of CD44 on the rearrangement of the actin cytoskeleton as conveyed by distinct cellular protrusions, we could prove that ACTBL2-positive TILs show a high co-expression of CD44, potentially hinting at a direct interaction in the course of leukocyte migration and activation.

Aiming at a particular identification of the predominant cell types and the accordingly conveyed tumor-modulating effects, additional staining analyses of the present EOC patient cohort defined the subset of

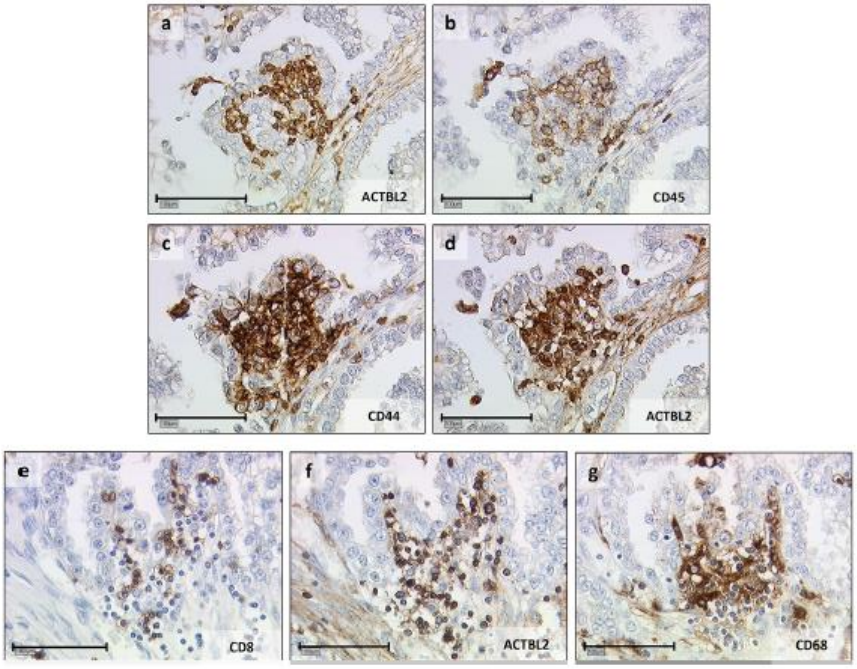

Figure 3. Serial staining of clear cell carcinoma tissue for leukocyte subtyping. (**a–d**) Exemplary photographs of consecutive clear cell carcinoma tissue slices after immunohistochemical staining of ACTBL2 (**a, d**), CD45 (**b**) and CD44 (**c**), identifying ACTBL2-overexpressing cells as tumor-infiltrating CD44-positive leukocytes. (**e–g**) Representative pictures of another clear cell carcinoma series, revealing CD8-positive cytotoxic T-cells (**e**) and CD68-positive macrophages (**g**) as the predominant cellular subsets of ACTBL2-positive TILs (**f**) (×25 magnification, scale bar = 100 µm).

ACTBL2-positive TILs as CD44-expressing cytotoxic T-cells (CD8+) and macrophages (CD68+). Besides being involved in the homing of leukocytes and antigen-presenting cells to sites of inflammation, CD44 was shown to directly mediate the lytic and anti-tumoral activity of cytotoxic T-cells by promoting specific transmembrane signals leading to granule exocytosis[47,48]. Further, Hegde et al. demonstrated that CD44 clustered at the contact between T-cells and mature DCs in the course of T-cell activation, mediating the formation of an immunological synapse[48]. The direct interaction of both cells resulted in an accumulation of F-actin within the T-cell at the specific binding point with a consecutive re-arrangement of the actin cytoskeleton, increasing the contact between the T-cell receptor and MHC-receptors on DCs[49]. Zhang et al. showed that the presence of intra-tumoral T-cells was an independent prognostic factor for overall and progression-free survival in ovarian cancer[50]. More specifically, intraepithelial CD8+ TILs and a high cytotoxic T-cell/regulatory T-cell (Treg) ratio were associated with a prognostic benefit for EOC patients[51]. Apart from the aforementioned mechanisms, tumor-associated macrophages (TAM) play a crucial role in shaping the distinct tumor environment by producing different cytokines and executing contrary functions depending on their polarization status[52,53]. Ovarian cancer patients with an increased M1/M2 TAM ratio in favor of tumor-suppressive and pro-inflammatory M1 macrophages showed a significantly improved overall survival[54,55]. However, ovarian cancer cells were shown to directly influence the polarization status of TAMs by changing it into the M2-like phenotype, which consequently is the predominant one in EOC and suppresses an effective cytotoxic T-cell response by fostering Treg recruitment[52,56,57]. In contrast, Paditaxel was shown to reprogram M2-polarized macrophages to the M1-like phenotype as a part of its therapeutic effect[58].

Taken together, our results suggest that the favorable prognostic effect of ACTBL2-expressing TILs in ovarian cancer is attributed to the presence and close interaction of activated cytotoxic T-cells and macrophages, probably in their function as antigen-presenting and T-cell-attracting cells. Additional analyses are required for a further distinction of the macrophage polarization status and the presence of dendritic cells in order to completely elucidate the definite cellular composition and interplay. As ACTBL2 and CD44, executing a key role in focal adhesion and T-cell activation, were both shown to closely interact with gelsolin in the course of cytoskeleton alteration, ACTBL2 might represent a novel marker for migrating and activated immune cells. Since sufficient tumor infiltration by T-cells is mandatory for a successful immune checkpoint blockade[59], the identification of

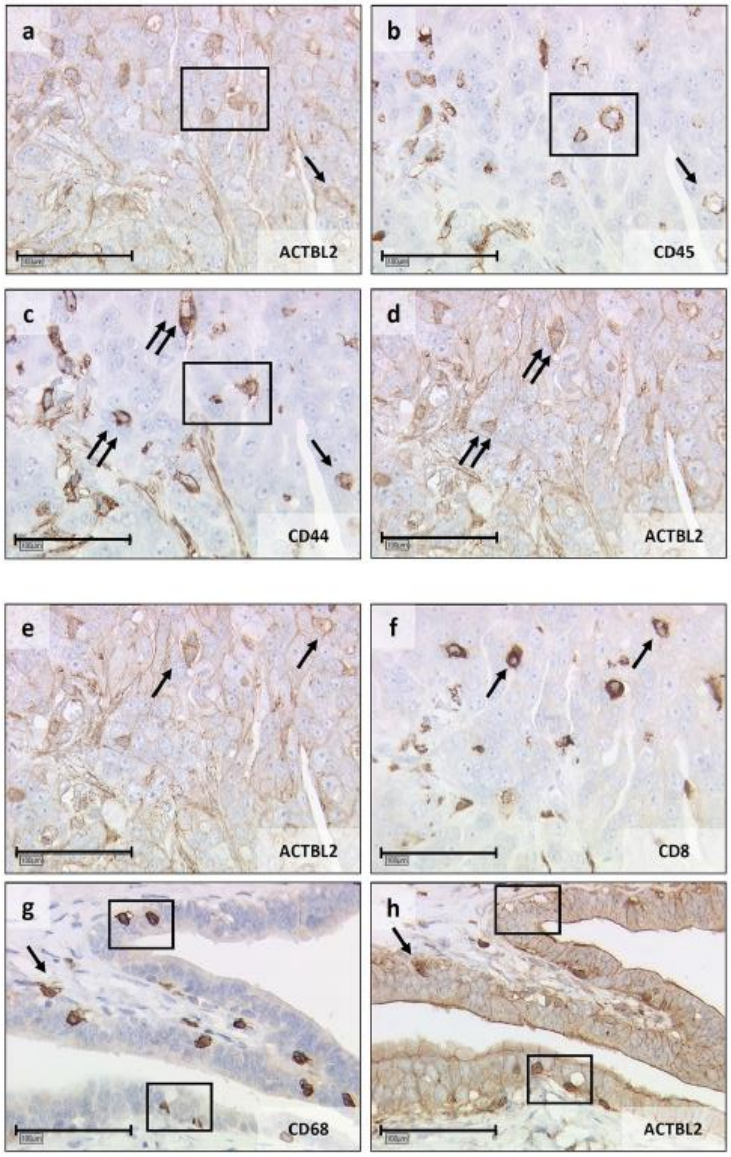

Figure 4. Serial staining of serous fallopian tube cancer for leukocyte subtyping. **(a–d)** Representative photographs of an immunohistochemical staining series of serous fallopian tube cancer, hinting at the co-expression of CD45 (**b**) and CD44 (**c**) by ACTBL2-positive (**a, d**) immune cells. **(e–h)** Exemplary pictures of two further series, identifying ACTBL2-overexpressing leukocytes (e and h) as CD8-positive (**f**) and CD68-expressing (**g**) immune cells. Identical cells between the pictures were marked by rectangles and arrows (×25 magnification, scale bar = 100 µm).

ACTBL2-positive CD44 + /CD8 + TILs in EOC tissue might be a means to determine potential patient subgroups, being particularly prone to respond to immune therapy.

Interestingly, correlation analyses showed a particular survival benefit for patients with LGSC and ACTBL2-positive TILs despite a low number of cases. Kurman et al. postulated a dualistic model, claiming that LGSC is a separate tumor entity due to specific histological characteristics and substantial molecular differences[7,30,60]. Patients with LGSC are characterized by a younger age at diagnosis and a better overall survival despite showing a relative resistance against platinum-taxane-based therapy due to its lower mitotic rate, warranting alternative and subtype-specific treatment strategies[61,62]. Since the majority of LGSC shows a positive estrogen receptor expression, hormone-based maintenance therapy revealed a promising effect on median progression-free survival compared to patients only undergoing clinical observation[63]. Other therapeutic options include the use of CDK4/6 inhibitors[61] and MEK inhibitor Trametinib for recurrent disease since the MAP-kinase pathway was shown to be a crucial part of LGSC pathogenesis[64,65]. To date, comprehensive analyses regarding the tumor microenvironment of LGSC considering the use and efficacy of immune checkpoint inhibition are missing, presumably because of its comparably rare occurrence. Milne et al. stated that the presence of TILs varied significantly between different subtypes of EOC, with HGSC showing the highest frequency of intra-tumoral CD45 + cells, but without taking LGSC into consideration[66]. Lacking TP53 or BRCA1/2 mutation and genomic stability further contribute to a reduced neo-antigen presentation in LGSC[67]. Additional detailed examinations of a larger number of cases are required to provide further knowledge on the unique composition of the tumor environment of LGSC and to elucidate the potential of immune therapeutical approaches.

Concluding, our study provides for the first time significant evidence of the favorable prognostic impact of ACTBL2-expressing TILs in epithelial ovarian cancer with regard to different histological subtypes. Actin beta-like 2 as a new and additional actin isoform may execute crucial functions in terms of leukocyte motility and activation by interacting with CD44 during cytoskeleton reorganization. Complementary studies are required to further analyze the role of ACTBL2 as a putative marker for activated migrating leukocytes on a molecular basis and to characterize the immunological impact of ACTBL2-positive TILs on ovarian carcinogenesis.

Data availability

The original contributions presented in the study are included in the article/the corresponding Supplementary Material. Further inquiries can be directed to the corresponding author.

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Author contributions

N.T. participated in conception and coordination of the study, performed the experiments, the statistical analysis and the visualization of the data and wrote the main part of the manuscript. B.C. and U.J. designed the study, participated in its coordination and analysis and approved the final version of the manuscript. D.M. provided essential material in the form of patients' tissue and participated in the design of the study by supervising immunohistochemistry and -fluorescence as a gynecologic pathologist. Further validation and formal analysis of the data was executed by D.M., C.K., C.S. and S.Ma. D.M., C.K., C.S., A.L., F.K., C.T., S.B., S.Me., A.H., T.K., A.B., S.Ma., F.T. and M.K. revised the manuscript for important intellectual content. All authors analyzed and interpreted the results and approved the final version of the manuscript.

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Competing interests

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Additional information

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