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Institut für Allgemeine Pathologie und Pathologische Anatomie

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Identifizierung prädiktiver und prognostischer Biomarker in unterschiedlichen Tumorkompartimenten des ösophagealen Adenokarzinoms

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Erklärung zum eigenen Anteil an den Publikationen

Die dieser Arbeit zugrunde liegenden Publikationen wurden im Rahmen von unterschiedlichen Forschungsprojekten am Institut für Pathologie des Universitätsklinikums Köln erstellt. Der eigene Anteil wird im Folgenden für die einzelnen Projekte beschrieben.

1. Anteil an der Publikation „Cell type-specific transcriptomics of esophageal adenocarcinoma as a scalable alternative for single cell transcriptomics“:

Die Entwicklung der unterschiedlichen Arbeitsschritte zu einem kontinuierlichen Protokoll und das Design des vorgestellten Verfahrens erfolgte durch mich selbst in Zusammenarbeit mit Herrn Prof. Dr. Axel Hillmer.

Die zugrunde liegenden Voruntersuchungen in Zellkultur-Experimenten sowie die Evaluierung alternativer Verfahren zur Zellsortierung (MACS Sorting) wurden durch mich selbstständig durchgeführt.

Die in der Publikation vorgestellten Verfahren zur Gewebedissoziation und die Immunfluoreszenz-Färbung wurden durch mich durchgeführt und für EAC-Biopsien optimiert, mit Unterstützung durch Herrn PD Dr. Patrick Plum.

Die flowzytometrische Sortierung der Zellen erfolgte im Max Planck Institute for Biology of Ageing gemeinsam mit Kat Folz-Donahue, welche für die technische Durchführung verantwortlich war. Gemeinsam Herrn PD Dr. Patrick Plum bereitete ich dabei die Proben für die Sortierung vor und legte zusammen mit Herrn Prof. Dr. Axel Hillmer die Gating Strategie fest.

Die RNA Extraktion der sortierten Zellproben erfolgte selbstständig durch mich mit Unterstützung von Herrn PD Dr. Patrick Plum.

Die Sequenzierung der RNA wurde durch Mitarbeiter des Cologne Center for Genomics durchgeführt. Die bioinformatische Aufbereitung der Ergebnisse führte Herr Dr. Oscar Velazquez Camacho durch.

Die Interpretation der Daten erfolgte durch mich selbst in Zusammenarbeit mit Herrn Prof. Dr. Axel Hillmer, Herrn PD Dr. Patrick Plum und Herrn Prof. Dr. Alexander Quaas.

Zu der Anfertigung der Manuskripts haben neben meiner Person Herr Prof. Dr. Axel Hillmer und Herr PD Dr. Patrick Plum zu gleichen Teilen beigetragen.

2. Anteil an den Publikationen „The expression of the immune checkpoint regulator VISTA correlates with improved overall survival in pT1/2 tumor stages in esophageal adenocarcinoma“, „Lymphocyte activation gene-3 (LAG3) mRNA and protein expression on tumour infiltrating lymphocytes (TILs) in oesophageal adenocarcinoma.“ und „Indoleamine 2.3-dioxygenase (IDO) expression is an independent prognostic marker in esophageal adenocarcinoma“:

Die in diesen Arbeiten durchgeführten immunhistochemischen und RNAScope basierten Untersuchungen wurden von mir selbst mit Unterstützung der medizinisch-technischen Assistentin Frau Wiebke Jeske durchgeführt.

Die in dieser Arbeit angegebenen mikroskopischen Auswertungen sind nach entsprechender Anleitung durch mich selbst unter der Supervision von Herrn Prof. Dr. Alexander Quaas und Frau Dr. Heike Löser durchgeführt worden.

Die retrospektiven Patientendaten wurden mir anonymisiert übermittelt und vollständig von mir selbst mit Unterstützung von Prof. Dr. Florian Gebauer ausgewertet.

Zu der Anfertigung der Manuskripte über die Immuncheckpoints IDO und VISTA haben neben meiner Person Herr Prof. Dr. Alexander Quaas, Herr Dr. Philipp Lohneis und Frau Dr. Heike Löser zu gleichen Teilen beigetragen. Zu der Anfertigung des Manuskripts über den Immuncheckpoint LAG3 haben neben meiner Person Herr Prof. Dr. Alexander Quaas, Herr Prof. Dr. Florian Gebauer und Frau Dr. Heike Löser zu gleichen Teilen beigetragen

3. Anteil an den Publikationen „HER2/neu (ERBB2) expression and gene amplification correlates with better survival in esophageal adenocarcinoma.“ und „PIK3CA and KRAS Amplification in Esophageal Adenocarcinoma and their Impact on the Inflammatory Tumor Microenvironment and Prognosis“

In den genannten Publikationen führte ich Teile der Auswertung der immunhistochemischen Untersuchungen durch und unterstützte zudem die Analyse und Diskussion der entsprechenden Ergebnisse.

Des Weiteren trug ich zur Erstellung der Manuskripte durch Anmerkungen zur Methodik und Korrektur der Ausführungen bei, wobei diese insbesondere durch Herrn Prof. Dr. Alexander Quaas, Frau Dr. Heike Löser, Frau Ahlem Essakly und Prof. Dr.

Florian Gebauer (PIK3CA) bzw. Herrn Prof. Dr. Alexander Quaas, Herrn PD Dr. Patrick Plum, Frau Dr. Heike Löser und Prof. Dr. Florian Gebauer (HER2/neu) erstellt wurden.

4. Anteil an der Publikation „Immune profile and immunosurveillance in treatment-naive and neoadjuvantly treated esophageal adenocarcinoma."

Für die genannte Publikation führte ich die Prozessierung der Formalin-fixierten Präparate im Bereich der RNA Extraktion durch, wobei die weitere Bearbeitung insbesondere auch durch Frau Svenja Wagener-Ryczek und Herrn Maximilian Schömmel erfolgte.

Des Weiteren trug ich zur Erstellung des Manuskripts durch Anmerkungen zur Diskussion von Immuncheckpoint-Inhibitoren und Korrektur der Ausführungen bei. Das Manuskript wurde hauptsächlich durch Frau Svenja Wagener-Ryczek, Herrn Maximilian Schömmel und Herrn Prof. Dr. Alexander Quaas erstellt.

Inhaltsverzeichnis

1. Abkürzungsverzeichnis

AEG	Adenokarzinom des gastroösophagealen Übergangs
Akt	Proteinkinase B
ARID1A	AT-Rich Interaction Domain 1A
Bcl2	B-Zell-Lymphom 2
BE	Barrett Ösophagus
C7	Complement component 7
CAF	Cancer Associated Fibroblast
CD	Cluster of Differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CNV	Copy Number Variation
CPS	Combined Positivity Score
CT	Computertomografie
CTL	Zytotoxischer T-Lymphozyt
CTLA-4	Cytotoxic T-Lymphocyte-associated Protein-4
CXCL5	C-X-C motif chemokine 5
CXCR4	CXC-Motiv-Chemokinrezeptor 4
DEG	Differentiell exprimierte Gene
DNA	Desoxyribonukleinsäure
DNA-Seq	DNA Sequenzierung
EAC	Esophageal Adenocarcinoma
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMA	Epithel Membrane Antigen
EMR	Endoskopische Mukosaresektion
EMT	Epithelial-Mesenchymale-Transition
EpCAM	Epithelial Cell Adhesion Molecule
ErbB Rezeptor	Epidermal Growth Factor Tyrosinkinase Rezeptoren
ERK	Extracellular Signal-Regulated Kinase
ESCC	Esophageal Squamous Cell Carcinoma

ESD	Endoskopische Submukosaresektion
EUS	Endoskopischer Ultraschall
EZM	Extrazelluläre Matrix
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FGFR2	Fibroblast Growth Factor 2
FISH	Fluorescence In Situ Hybridization
FNP	Feinnadelpunktion
Foxp3	Forkhead-Box-Protein P3
GERD	Gastroösophageale Refluxerkrankung
HB-EGF	Heparin Binding Epidermal Growth Factor
HER	Human Epidermal Growth Factor Receptor
HCC	Hepatozelluläres Karzinom
HIF1α	Hypoxia-Inducible Factor 1α
HLA	Humanes Leukozyten Antigen
IC-Score	Immune Cell Score
ICI	Immuncheckpoint-Inhibitor
IDO	Indolamin-2,3-Dioxygenase
IFNγ	Interferon-γ
IL	Interleukin
IL1RN	Interleukin-1 Receptor Antagonist
JAK	Janus Kinase
KRAS	Kirsten Rat Sarcoma Viral Oncogene
LAG3	Lymphocyte Activation Gene 3
LILRB1	Leukocyte Immunoglobulin Like Receptor B1
M	männlich
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MEK	MAPK/Erk kinase
MET	MET proto-oncogene
MDCT	Multidetektor-Computertomografie

MDSC	Myeloische Suppressorzelle
MHC	Haupthistokompatibilitätskomplex
mRNA	messenger Ribonukleinsäure
mTOR	Mechanistic Target of Rapamycin Kinase
NF-κB	Nuclear Factor-'kappa-light-chain-enhancer' of activated B-cells
NGS	Next Generation Sequencing
NK Zellen	Natürliche Killerzellen
NSCLC	Nicht kleinzelliges Lungenkarzinom
PCA	Principal Component Analysis
PDGF	Platelet Derived Growth Factor
PDGFRA	Platelet Derived Growth Factor Subunit A
PD-1	Programmed Cell Death Protein-1
PD-L1	Programmed Cell Death Ligand-1
PET	Positronen-Emissionstopografie
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit α
qPCR	quantitative-Echtzeit-Polymerase-Kettenreaktion
Raf	Raf-1 proto-oncogene
RNA	Ribonukleinsäure
RNA-Seq	RNA Sequenzierung
scRNA-Seq	Single Cell RNA Sequencing
SHP2	Src Homology region 2 domain-containing Phosphatase-2
SMAD4	SMAD Family Member 4
STAT	Signal Transducer and Activator of Transcription
TCGA	The Cancer Genome Atlas
TGF	Transformierender Wachstumsfaktor
TH Zelle	T-Helferzelle
TIL	Tumor Infiltrierender Lymphozyt
TIM-3	T-cell Immunoglobulin Mucin Family Member 3
TME	Tumor Microenvironment, Tumormikromilieu
TNF α	Tumor Nekrose Faktor α
TNFRSF1	TNF Receptor Superfamily Member 17

TPS	Tumor Proportion Score
TP53	Tumor Protein 53
Treg	regulatorischer T-Lymphozyt
UICC	Union Internationale Contre le Cancer
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VISTA	V-domain Ig Suppressor of T cell Activation
W	weiblich

2. Einleitung

2.1 Das Adenokarzinom des Ösophagus

2.1.1 Epidemiologie

Das Ösophaguskarzinom stellt global betrachtet die acht häufigste Krebsart mit der sechsthöchsten krebsassoziierten Mortalität dar, wobei die 5-Jahres-Überlebensrate weniger als 25% beträgt [1]. Weltweit wurden im Jahr 2020 604.100 Neuerkrankungen sowie 544.076 Todesfälle registriert [2]. In Deutschland erkrankten im Jahr 2016 5.540 Männer sowie 1.740 Frauen an Ösophaguskarzinomen bei einem mittleren Erkrankungsalter von 67 (m) bzw. 71 (w) Jahren [3].

Während das Plattenepithelkarzinom (Esophageal Squamous Cell Carcinoma, ESCC) weltweit gesehen aufgrund hoher Inzidenzen im asiatischen Raum den häufigsten histologischen Subtyp darstellt, ist das Adenokarzinom (Esophageal Adenocarcinoma, EAC) in den USA, Australien sowie vielen westlichen europäischen Ländern (z.B. Frankreich und die Niederlande) inzwischen der führende Subtyp [4]. In Deutschland lag der Anteil des EACs nach Angaben des Zentrums für Krebsregisterdaten im Jahr 2016 bei 48% gegenüber 43% ESCCs bei Männern, hingegen bei Frauen lediglich bei 34% EACs gegenüber 56% ESCCs [5].

Weltweit wird bis zum Jahr 2030 ein Anstieg von 35% in der Inzidenzrate von Ösophaguskarzinomen gegenüber dem Jahr 2018 erwartet [6]. Während die Inzidenzrate des ESCCs hierbei in den meisten Ländern sinkt bzw. sich stabilisiert, zeigt sich global durchgehend ein deutlicher Anstieg dieser für das EAC [7]. Regionale Unterschiede sind hierbei von großer Bedeutung – so wird in Japan beispielsweise entgegen dem weltweiten Trend eine weitere Zunahme der Inzidenz von ESCCs erwartet, wohingegen in den USA ein Anstieg des Anteils der EACs an der Gesamtheit der Ösophaguskarzinome auf bis zu 75% prognostiziert wird [7].

2.1.2. Ätiologie und Pathogenese

Risikofaktoren für die Entstehung eines EACs sind das Vorliegen einer gastroösophagealen Refluxerkrankung (GERD), das Vorliegen eines Barrett-Ösophagus (BE), darüber hinaus Übergewicht, Tabakkonsum, geografische Aspekte (Nordamerika, Westeuropa, Australien als Regionen mit erhöhter Inzidenz), ethnische

Gruppenzugehörigkeit (weiße Menschen ca. fünf Mal häufiger betroffen als schwarze Menschen), nutritive Gewohnheiten (wenig Aufnahme von Obst und Gemüse) sowie das männliche Geschlecht [8-10].

Ein Großteil der EACs entsteht auf dem Boden eines sogenannten Barrett-Ösophagus [11]. Hierbei handelt es sich um eine Metaplasie des ösophagealen Plattenepithels in dünndarmartiges Zylinderepithel als Folge einer chronischen Belastung durch Magensäure. Dabei spielen wahrscheinlich pluripotente Basalzellen am gastroösophagealen Übergang eine entscheidende Rolle, in dem sie geschädigtes Plattenepithel durch Zylinderepithel ersetzen [12, 13]. Die genaue Pathophysiologie der Barrettmucosa ist aber nicht geklärt. Im weiteren Verlauf kann sich im Rahmen der sogenannten Metaplasie-Dysplasie-Karzinom Sequenz ein EAC entwickeln [14]. In etwa 5-15% wird bei endoskopischen Kontrollen eines symptomatischen Refluxes ein BE nachgewiesen [15]. Das jährliche Risiko der malignen Transformation eines BEs in ein EAC beträgt 0,1-0,5% [12, 15].

Gegenstand aktueller Forschung ist die Identifizierung möglicher Treiber-Gene, welche die Entstehung des EACs begünstigen und als Angriffspunkte einer zielgerichteten Therapie dienen könnten. Hierbei zeigen TCGA (The Cancer Genome Atlas) Daten sowie weiterführende genomische Studien unter anderem gehäuft Mutationen der Tumorsuppressor-Gene TP53, CDKN2A und SMAD4 sowie des Chromatin-Remodelling-Faktors ARID1A [13, 16-19]. Frankell et al. finden überdies in einem Kollektiv von 551 EAC Patienten Mutationen und Amplifikationen in den Genen KRAS und ERBB2 (Genprodukt: HER2/neu), welche potenzielle Onkogene aus Epidermal Growth Factor Tyrosinkinase Rezeptor (ErbB-Rezeptoren) abhängigen Signalkaskaden darstellen [16]. HER2/neu amplifizierte EACs bilden nach heutigem Stand den einzigen molekularen Subtyp mit therapeutischer Konsequenz [20].

2.1.3 Histologie

Das Adenokarzinom stellt neben dem Plattenepithelkarzinom den häufigsten histologischen Subtyp des Ösophaguskarzinoms dar [21]. Es entsteht in den meisten Fällen im distalen Ösophagus, selten auch weiter proximal bei heterotoper Magenschleimhaut in diesem Gebiet [22]. Entsprechend der oben beschriebenen Pathogenese auf dem Boden einer dünndarmartig veränderten Barrett Mukosa zeigt sich histologisch typischerweise das Bild glandulärer Strukturen [23]. Diese sind abhängig vom Differenzierungsgrad in über 95% (G1), 50-95% (G2) bzw. unter 50%

(G3) ausgeprägt [23]. Schlecht differenzierte EACs sind durch kaum erkennbare, irreguläre Drüsenverbände bzw. Nester atypischer, einzeln liegender oder solide gelagerter Karzinomzellen charakterisiert [23].

2.1.4 Klinik

Das EAC führt in der Regel erst in fortgeschrittenen Tumorstadien zu Symptomen, was die zumeist eingeschränkten therapeutischen Optionen begründet [24]. Die Patienten stellen sich häufig mit Dysphagie, rezidivierendem Erbrechen unverdauter Nahrung und Gewichtsverlust vor, zudem mit unspezifischen retrosternalen Beschwerden [21, 25, 26]. Zusätzlich können Zeichen einer gastrointestinalen Blutung entsprechend einer anämischen Symptomatik, Teerstuhl und Hämatemesis vorliegen [25]. Im Rahmen einer lymphogenen Metastasierung zeigen sich zervikale Lymphadenopathien [27]. Darüber hinaus deuten jeweils organspezifische Symptome auf eine mögliche hämatogene Metastasierung hin [27]. Diese findet sich zumeist in der Leber, den Knochen, der Lunge, den Nebennieren sowie im Gehirn [28].

2.1.5 Diagnostischer Algorithmus

Für den anamnestischem Verdacht existiert zur weiteren Abklärung ein festgelegter Algorithmus in der S3-Leitlinie zur Diagnostik und Therapie der Plattenepithelkarzinome und Adenokarzinome des Ösophagus [26]. Dieser beinhaltet eine initiale Endoskopie mit Biopsieentnahme zur morphologischen Sicherung der Diagnose als Goldstandard. Im weiteren Verlauf erfolgt die Festlegung des klinischen TNM-Stadiums (Staging): Hierbei wird ein endoskopischer Ultraschall (EUS) zur Untersuchung der Eindringtiefe (T-Stadium) und Detektion regionaler Lymphknotenmetastasen (N-Stadium) durchgeführt. Ggf. wird dieser erweitert durch eine Feinnadelpunktion (FNP) zur morphologischen Sicherung letzterer. Der EUS soll durch eine kontrastmittelgestützte Multidetektor-Computertomografie (MDCT) von Thorax und Abdomen ergänzt und die Befunde aus EUS+MDCT gemeinsam interpretiert werden. Zum Ausschluss einer Lebermetastasierung erfolgt zudem standardmäßig eine abdominelle Ultraschalluntersuchung. Bei positivem Nodalstatus (cN+) und/oder einer Eindringtiefe über die Submukosa hinaus (cT 2-4) kann eine Positronen-Emissionstopografie (PET) mit einem CT kombiniert werden (PET/CT) zur genauen Beurteilung des M-Stadiums.

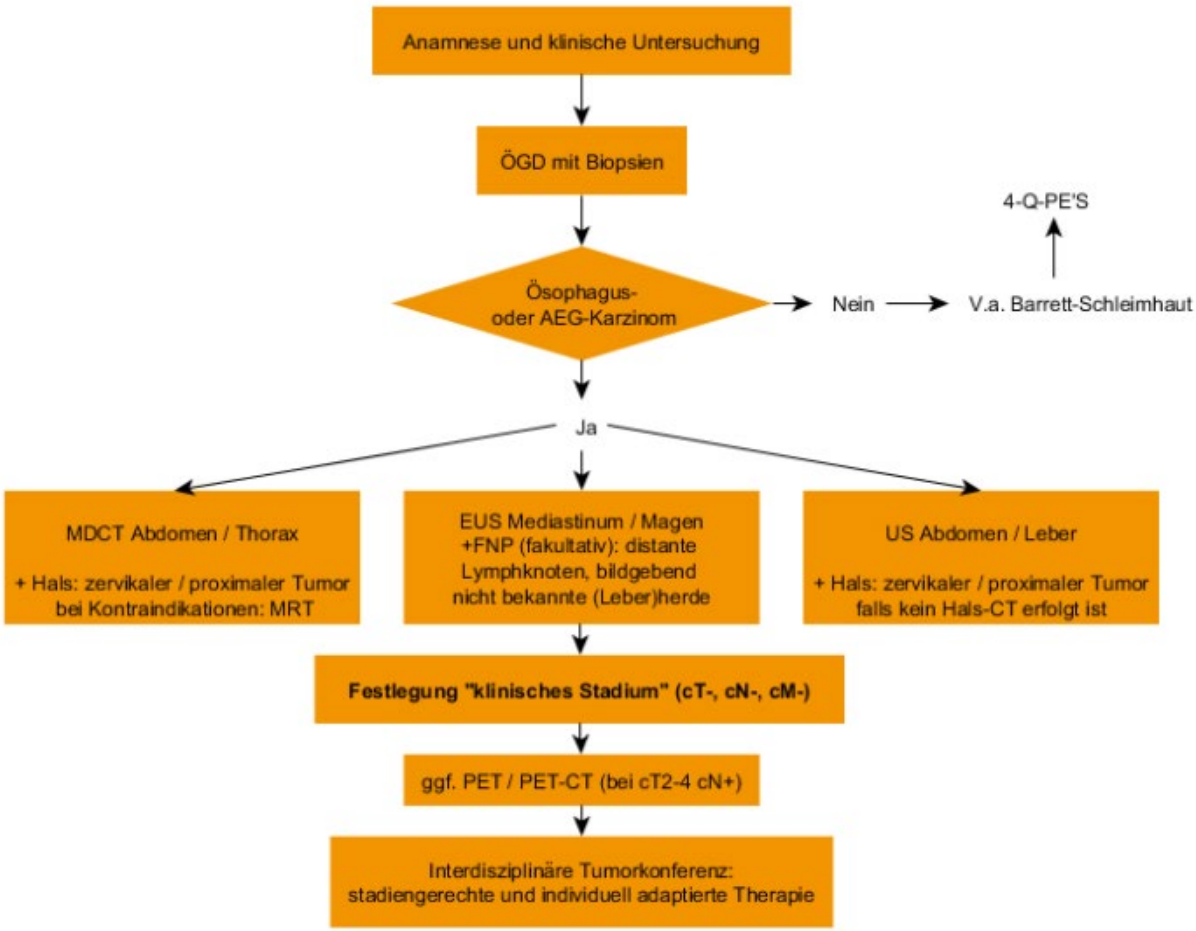

Abbildung 1 (S3-Leitlinie zur Diagnostik und Therapie der Plattenepithelkarzinome und Adenokarzinome des Ösophagus, AWMF, 2019 [26])

Diagnostischer Algorithmus beim Ösophaguskarzinom

2.1.6 Therapie

Die Therapie des EACs umfasst unterschiedliche Modalitäten, welche je nach Tumorstadium und individueller Verfassung des Patienten zum Einsatz kommen. Dabei stellt die TNM Klassifikation eine entscheidende Grundlage zur Festlegung des Therapiepfades dar. Grundsätzlich kommen lokal begrenzte EACs (cTx, cNx, cM0) für eine kurative Therapie infrage. Beim Vorliegen von Fernmetastasen oder lokal stark fortgeschrittenem Befund wird hingegen nach aktueller Leitlinie ein palliatives Konzept empfohlen [26].

2.1.6.1 Kurative Therapie

Im Stadium cT1 kann ein endoskopisches Verfahren zum Abtragen der Läsion mittels der sogenannten endoskopischen Mukosaresektion (EMR) bzw. der endoskopischen Submukosaresektion (ESD) erfolgen [26, 29]. Spätestens ab dem Stadium T2, ebenso aber im Stadium cT1 beim Vorliegen von Risikofaktoren (Lymph- und/oder Veneninvasion, schlechter Differenzierungsgrad (≥G3), tiefe Submukosainfiltration ≥500 µm) wird ein operatives Verfahren empfohlen [26]. Als chirurgische Standardtherapie gilt hierbei die abdominothorakale, subtotale Ösophagusresektion mit 2-Feld-Lymphknotendissektion und Magenhochzug. Als alternatives Verfahren kommt bei Adenokarzinomen des gastroösophagealen Übergangs (AEG) eine transhiatale Resektion als erweiterte Gastrektomie zur operativen Sanierung infrage [26, 30]. Obwohl beim Vorliegen von Fernmetastasierung in der Leitlinie eine Operation nicht empfohlen wird, untersuchen aktuelle Phase III Studien bei Patienten mit wenigen Metastasen (sogenannte „Oligometastasen“) den Stellenwert einer kurativen Behandlungsstrategie durch Resektion des Primärtumors mit gleichzeitiger Entfernung der Metastasen (RENAISSANCE/FLOT5-Studie, GASTRIPEC-Studie) [26, 30-32].

Ab dem Stadium cT2 kann und ab dem Stadium cT3 sollte zudem eine multimodale Therapie erfolgen [26]. Nach aktueller Leitlinie sind hierbei eine perioperative Chemotherapie (FLOT-Schema: 5-Fluorouracil, Folinsäure, Oxaliplatin, Docetaxel) und eine neoadjuvante kombinierte Radio-Chemotherapie (CROSS-Schema: Paclitaxel, Carboplatin und 41,4 Gy in 23 Fraktionen) als gleichwertig anzusehen [26]. Aktuelle Phase III Studien vergleichen derzeit die beiden Konzepte, so etwa die irische Neo-AEGIS Studie und die deutsche ESOPEC Studie [33, 34].

Alternativ kann eine definitive Radio-Chemotherapie in kurativer Intention bei Inoperabilität des Tumors erfolgen. Dies gilt ebenso für den Fall, dass der Patient funktionell nicht operabel ist oder die Operation ablehnt [26].

2.1.6.2 Palliative Therapie

Bei lokal stark fortgeschrittenem Befund oder dem Vorliegen von Fernmetastasen empfiehlt die Leitlinie eine palliative Systemtherapie mit Platin-haltigen (Oxaliplatin oder Cisplatin) sowie Fluoropyrimidin-haltigen Substanzen (5-FU oder Capecitabin). Diese sollen in einer Zwei- oder Dreifachkombination eingesetzt werden [26]. Überdies

kann hier bei HER2/neu überexprimierenden EACs der zusätzliche Einsatz des Antikörpers Trastuzumab erwogen werden [26]. Neben weiterer Chemotherapie ist als weitere zielgerichtete Therapieoption in der aktuellen Leitlinie lediglich der VEGFR Antikörper Ramucirumab als Zweitlinientherapie im palliativen Konzept genannt. Die Empfehlung ergibt sich aus Überlebensvorteilen von ca. zwei Monaten in zwei größeren Studien zu fortgeschrittenen Magenkarzinomen und Adenokarzinomen des gastroösophagealen Übergangs (REGARD bzw. RAINBOW Studie) [35, 36].

Neben der palliativen Systemtherapie stellen lokale Bestrahlung oder Brachytherapie weitere Möglichkeiten zur Symptomlinderung im Rahmen der Palliation dar [26].

2.1.7 Prognose

Das EAC hat mit einer globalen 5-Jahren-Überlebensrate von weniger als 25% eine schlechte Prognose [1, 37]. Für Deutschland liegen keine getrennten Daten für ESCC und EAC vor: Im Jahr 2016 war das 5-Jahres-Überleben des Ösophaguskarzinoms mit 24% (w) bzw. 22% (m) mit den internationalen Zahlen vergleichbar [5].

Aufgrund der unspezifischen und insgesamten späten Symptompräsentation werden Patienten häufig in fortgeschrittenen Tumorstadien diagnostiziert [24]. Abhängig vom UICC Stadium zeigt sich eine sukzessive Verschlechterung des 5 Jahresüberlebens in höheren Stadien. So gibt beispielsweise das britische Office for National Statistics für die Jahre 2013-2017 ein solches von 52,8% (Stadium 1), 29,9% (Stadium 2), 16,3% (Stadium 3) bzw. 0% (Stadium 4) an (37.169 Patienten, Daten für ESCC und EAC gemischt) [38].

Die Bedeutung prädiktiver und prognostischer Marker für das EAC scheint in dem Kontext belastender und eingeschränkter Therapiekonzepte sowie schlechter Gesamtprognose umso wichtiger. So ist derzeit die HER2/neu Amplifikation der einzige prädiktive Marker mit therapeutischer Konsequenz, wobei die prognostische Bedeutung umstritten ist [20, 26, 39]. Daten der vor Kurzem publizierten Ergebnisse der CheckMate 649 Studie scheinen darüber hinaus eine prädiktive Bedeutung der Höhe der PD-L1 Expression für das Ansprechen auf eine Immuntherapie mit Nivolumab zu zeigen [40]. Eine mögliche Prognoserelevanz bleibt jedoch unklar [41, 42]. Weitere therapeutisch relevante Biomarker wie EGFR Amplifikation, MET Amplifikation und FGFR2 Amplifikation werden im Rahmen klinischer Studien untersucht [43].

2.2 Das Tumormikromilieu

2.2.1 Zusammensetzung und Funktion des Tumormikromilieus

Das Tumormikromilieu (*engl. tumor microenvironment*, TME) besteht, neben den malignen Tumorzellen selbst, aus einer großen Anzahl nicht-neoplastischer Zellen (u.a. Fibroblasten, Endothelzellen, Immunzellen, Adipozyten, glatte Muskelzellen) sowie der extrazellulären Matrix (EZM) [44, 45]. Eine einheitliche Struktur in der Beschreibung der unterschiedlichen Tumorkompartimente existiert dabei nicht. Eine mögliche Einteilung ergibt sich in Anlehnung an Pattabiraman et al., welche nicht-zelluläre (Extrazellulärmatrix) und zelluläre Bestandteile des Tumors unterschieden. Letztere werden weiter unterteilt: Die tumoralen Zellen stehen den nicht-tumoralen Zellen gegenüber, wobei diese entweder mesenchymalen (u. a. Fibroblasten, Adipozyten, Endothelzellen) oder hämatopoetischen (v.a. Immunzellen) Ursprungs sind [46].

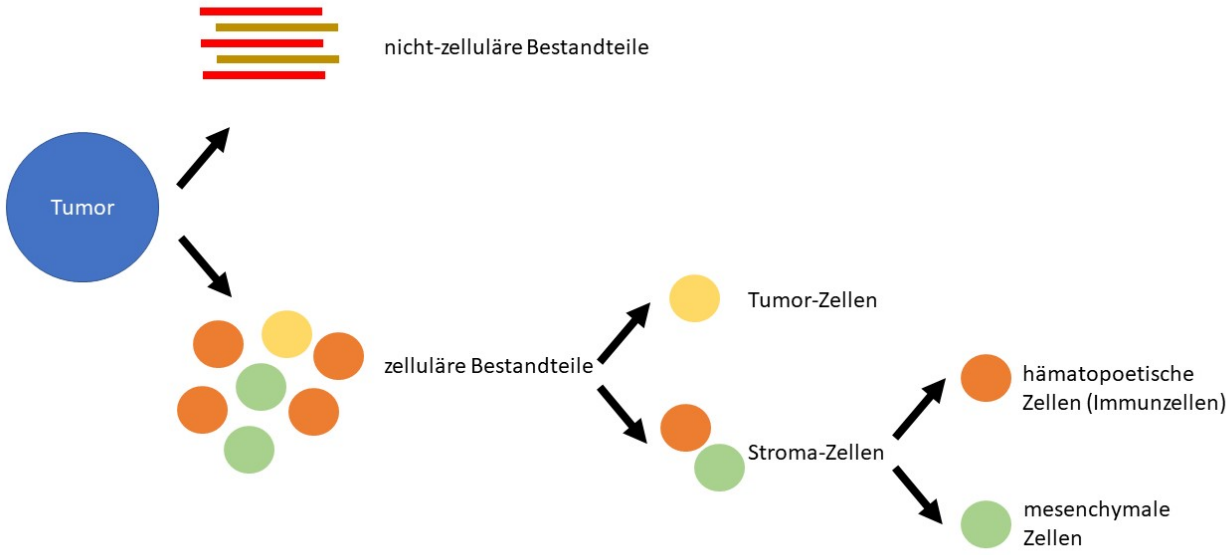

Abbildung 2: (nach Pattabiraman et al, Nature Reviews Drug Discovery, 2014 [46]) Einteilung des Tumormikromilieus

Die Bedeutung des Zusammenspiels der Tumorzelle mit dem Tumorstroma scheint dabei von entscheidender Bedeutung für die Pathogenese maligner Tumore zu sein,

möglicherweise sogar wichtiger als die Bedeutung der Tumorzellen selbst [47, 48]. Ein Beispiel für die enorme Relevanz der Interaktion zeigt sich etwa im gesteigerten Risiko zur malignen Entartung von chronisch entzündetem Gewebe: So entwickeln in Studien 27% der Patienten mit einer Leberzirrhose binnen 12 Jahren ein hepatozelluläres Karzinom (HCC) und Patienten mit lang andauernder chronisch entzündlicher Darmerkrankung (>10 Jahre) zeigen ein 7-fach erhöhtes Risiko für die Entstehung eines Kolonkarzinoms [48-50]. Hierbei führen wahrscheinlich fehlregulierte Reaktionen im Stroma zur Tumorprogression bzw. Initiation der Karzinogenese [48]. Das TME beeinflusst somit die Entstehung und Prognose eines Tumors und kann darüber hinaus auch wertvolle Hinweise für die zu erwartende Effektivität einer Therapie liefern [44].

Die unterschiedlichen Mechanismen des Zusammenspiels von Tumor- und Stromazellen sind komplex. Studien fokussieren sich daher häufig auf einzelne Aspekte dieser Kommunikation [48]. Grundsätzlich beeinflussen sich die Komponenten jedoch gegenseitig über Zytokine, Wachstumsfaktoren und Matrixenzyme [51]. Stromazellen führen über die Ausschüttung solcher zur Aktivierung von Transkriptionsfaktoren wie NF-κB (nuclear factor-κB), STAT 3 (signal transducer and activator of transcription 3) und HIF1α (hypoxia-inducible factor 1α) in der Tumorzelle [52]. Diese schüttet ihrerseits Zytokine, Chemokine und Prostaglandine aus, was wiederum zur Rekrutierung weiterer Stromazellen führt [52]. In den Stromazellen werden nun wie schon in der Tumorzelle Transkriptionsfaktoren aktiviert und es kommt im Verlauf zur Generierung eines tumorgenen Mikroenvironments durch gegenseitige Stimulation [52].

Dieser Mechanismus weist dabei erstaunliche Parallelen zum physiologischen Wundheilungsprozess auf [51]. Auch hier kommt es zur Einwanderung von Leukozyten und Thrombozyten durch Ausschüttung von Chemokinen zum Ort der Gewebeschädigung [53]. Zudem werden Wachstumsfaktoren und Zytokine zur Zellproliferation und Neoangiogenese ausgeschüttet (z.B. PDGF, VEGF, FGF, TGFβ) und es kommt zum Umbau der Extrazellulärmatrix [53]. Während diese interaktiven Prozesse im Rahmen der Wundheilung kontrolliert und zeitlich begrenzt stattfinden, verlaufen sie in der Tumorentstehung unkontrolliert.

2.2.2 Die Darstellung des Tumormikromilieus im Rahmen dieser Promotion

Die vorliegende Arbeit fasst eine Anzahl an Publikationen zu unterschiedlichen Themenbereichen des TMEs beim EAC zusammen. Ihr gemeinsames Ziel liegt in der Identifizierung prognostischer und prädiktiver Biomarker in verschiedenen Tumorkompartimenten. Zum einen wurde hierbei ein neues Verfahren zur Identifizierung Kompartiment-spezifischer Biomarker mittels Transkriptomanalyse von flowzytometrisch-sortierten Gewebeproben etabliert. Darüber hinaus wurden konkrete Biomarker aus dem Immunkompartiment und dem Tumorzellkompartiment durch immunhistochemische und RNA-Sonden basierte Verfahren sowie Fluorescence In Situ Hybridization (FISH-Analyse) näher analysiert.

Inhaltlich gesehen lassen sich die Projekte somit drei Schwerpunkten zuordnen, welche zur besseren Strukturierung nacheinander in der Einleitung betrachtet werden:

1. Entwicklung einer Methode zur Identifizierung Kompartiment-spezifischer Biomarker (im mesenchymalen Zellkompartiment, im Tumorzellkompartiment und im Immunkompartiment)
2. Biomarker des Immunkompartiments und deren Dynamik unter Metastasierung und Chemotherapie
3. Biomarker in ErbB-Rezeptor abhängigen Signalwegen im Tumorzellkompartiment

2.3 Entwicklung einer Methode zur Identifizierung Kompartiment-spezifischer Biomarker

Der Fokus in der Forschung zu soliden Tumoren hat sich vom alleinigen Blick auf die häufig epithelialen Tumorzellen zu einer verstärkten Betrachtung weiterer Bestandteile des TMEs entwickelt [54]. Die Entdeckung modulierender Proteine der hämatopoetischen Immunzellen (Immuncheckpoints) hat bereits zur Etablierung von relevanten Therapieoptionen in zahlreichen Tumorentitäten geführt (vgl. Kapitel 2.4). Außerdem gibt es zunehmend Hinweise für den Einfluss von so genannten Cancer Associated Fibroblasts (CAFs) als Vertreter des mesenchymalen Zellkompartiments [55]. Zahlreiche auto- und parakrine Funktionen dieser sind beschrieben und ihre inflammatorisch wirksamen Zytokine und Wachstumsfaktoren beeinflussen das Tumorwachstum, Neovaskularisation sowie die Bildung eines immunsuppressiven

TMEs [54, 56]. Die genauere Charakterisierung von CAFs stellt sich z.B. aufgrund des Fehlens eindeutiger immunhistochemischer Marker oft schwierig dar [57, 58]. Dennoch ist ihr vermehrtes Auftreten in verschiedenen Tumorentitäten mit einer schlechten Prognose assoziiert [59-61]. Sowohl mesenchymale als auch epitheliale und hämatopoetische Zellen haben daher eine individuelle Bedeutung für das Tumorwachstum und müssen in Forschungsfragen und -methoden gleichsam berücksichtigt werden.

Durch die Etablierung des Next Generation Sequencings (NGS) konnten in der Vergangenheit wichtige Erkenntnisse für molekulare Grundlagen von Tumoren auf Ebene der Nukleinsäuren gewonnen werden [62]. Dabei ermöglicht die Sequenzierung der DNA (DNA-seq) Aussagen über das Genom der Zellen und die Sequenzierung der RNA (RNA-seq) über deren Genexpression bzw. deren Transkriptom [63]. Zahlreiche NGS-Studien fokussieren sich auf die Analyse ganzer Gewebeproben, welche unterschiedliche Populationen von Zellen enthalten (sog. Bulk Sequencing) [64]. Hierbei wird durch die gemischten Zelltypen die bioinformatische Analyse jedoch häufig erschwert [65-67]. Insbesondere die Daten aus selteneren Zellformen können beispielsweise leicht übersehen werden [65].

Um auch auf der Ebene einzelner Zellen Sequenzierungsdaten zu erhalten, wurde im Jahr 2009 erstmals die Methode des Single Cell Sequencings angewandt und seitdem weiter etabliert [64, 68]. Im Gegensatz zum Bulk Sequencing werden hierbei zunächst Einzelzellen durch unterschiedliche Methoden (u.a. Verdünnungsreihen, Laser-Microdissection, Fluorescence Activated Cell Sorting/FACS, mikrofluidische Plattformen) isoliert [64]. Anschließend wird die DNA bzw. RNA aus der Zelle extrahiert, amplifiziert und sequenziert [64]. Durch dieses Verfahren kann somit genau bestimmt werden, welcher Zelltyp für bestimmte Eigenschaften des Genoms bzw. Transkriptoms verantwortlich ist [68]. Ein Beispiel für den zusätzlichen Informationsgewinn zeigen u.a. Li et al. durch eine Single Cell Transkriptom Sequenzierung (scRNA-seq) von Gewebeproben des Kolonkarzinoms [69]: Sie beschreiben eine hochregulierte Genexpression der epithelial-mesenchymalen Transition (EMT) allein in CAFs und nicht wie vermutet in den epithelialen Zellen. Da CAFs als mesenchymale Zellen von Natur aus auch eine mesenchymale Genexpression zeigen, hätte eine Analyse des gesamten Transkriptoms durch Bulk

Sequencing fälschlicher Weise zur Annahme eines EMT-Expressionsprofils von Tumorzellen führen können [54].

Ungeachtet ihrer Vorteile und Möglichkeiten ist die Methode des Single Cell Sequencings jedoch mit hohen Kosten verbunden, so dass für repräsentative Studien oft die notwendige Anzahl an Patienten nur schwer erreicht werden kann [66]. Um Zelltyp-spezifische Alterationen der Genexpression beim EAC dennoch in einem größerem Patientenkollektiv nachzuweisen, sind daher neue methodische Ansätze erforderlich. Im Rahmen dieser Arbeit wurde daher ein Protokoll entwickelt, um unterschiedliche Zellkompartimente (epitheliale Zellen, Immunzellen, Fibroblasten) aus endoskopisch gewonnenem EAC-Gewebe und korrespondierender Mukosa zu isolieren und anschließend Zelltyp-spezifisch ihr Transkriptom zu sequenzieren. Das Ziel war es, hierdurch zum Single Cell Sequencing vergleichbare Aussagen zu generieren, ohne dabei auf eine RNA-Extraktion aus Einzelzellen angewiesen zu sein. Eine Analyse von Genexpressionsunterschieden zwischen normaler Ösophagus-Schleimhaut und Tumorgewebe kann zur Identifizierung Kompartiment-spezifischer Biomarker beitragen und Hinweise für die Bedeutung der einzelnen Zelltypen beim EAC ergeben.

2.4 Biomarker des Immunkompartiments und deren Dynamik unter Metastasierung und Chemotherapie

2.4.1 Die Zusammensetzung des Immunkompartiments

Das Immunkompartiment maligner Tumore besteht sowohl aus Teilen der angeborenen als auch der erworbenen Immunabwehr [70]. Hierbei stellen CD8 positive T-Lymphozyten, CD4 positive T-Lymphozyten, proinflammatorische Makrophagen des M1 Typs, natürliche Killer Zellen (NK Zellen) und dendritische Zellen wichtige Bestandteile der anti-tumoralen Immunantwort dar [70]. Dagegen haben myeloische Suppressorzellen (MDSCs), regulatorische T-Lymphozyten (Tregs) und Makrophagen des M2 Typs durch immunsuppressive Eigenschaften einen tumor-fördernden Effekt [70, 71]. Die verschiedenen Zelltypen interagieren dabei über Zytokine und Oberflächenproteine sowohl untereinander als auch mit den Tumorzellen (siehe Abbildung 3).

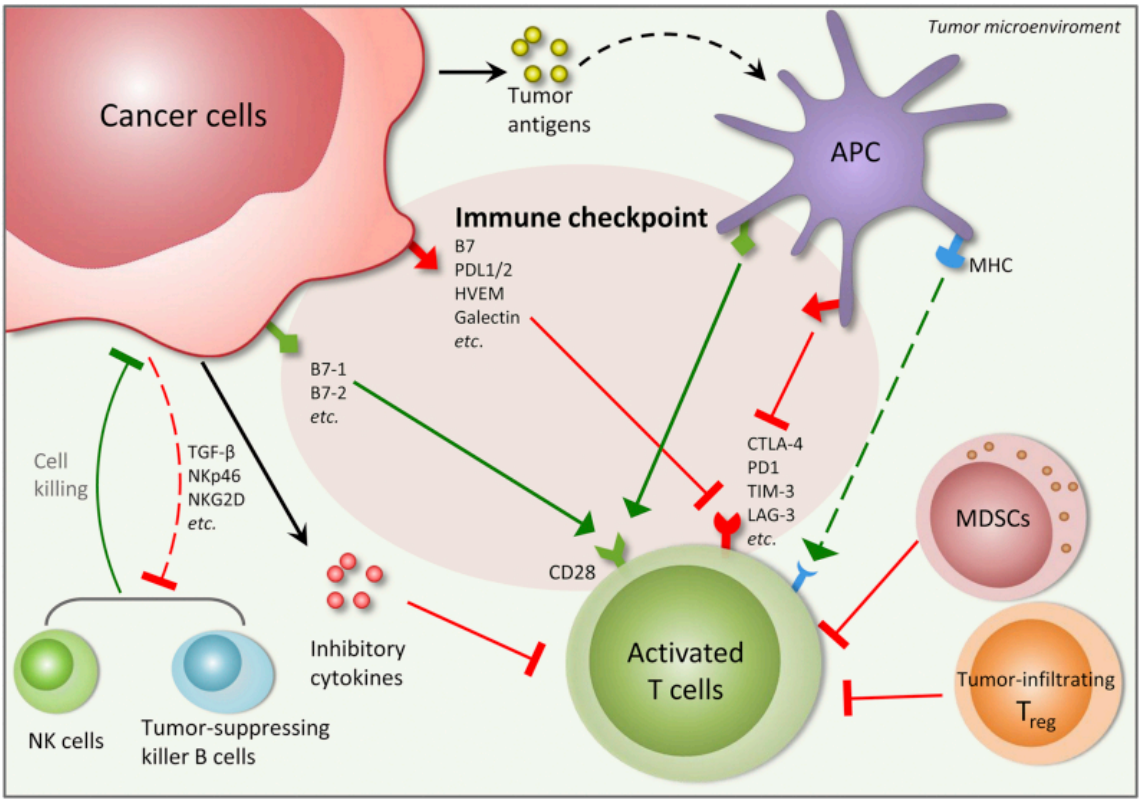

Abbildung 3: (Lin et al., Asian J Urol, 2016 [71])

Schematische Darstellung des Immunkompartiments

Insbesondere den CD3 positiven T-Lymphozyten (Hauptsubtypen: CD4 vs. CD8 positive Zellen) kommt eine besondere Bedeutung zu, weshalb hier kurz näher auf sie eingegangen werden soll [72]. Aktivierte, CD8 positive T Zellen induzieren nach Bindung an den Haupthistokompatibilitätskomplex Klasse 1 (MHC-I) von Tumorzellen eine Apoptose dieser und werden daher auch als zytotoxische T Lymphozyten (CTLs) bezeichnet [72]. Die Infiltration des Tumors mit CTLs stellt einen entscheidenden Aspekt in der Tumorkontrolle durch das Immunsystem dar [73]. CD4 positive T-Lymphozyten werden dagegen nach Antigenpräsentation durch den Haupthistokompatibilitätskomplex Klasse 2 (MHC-II) aktiviert [72]. Dieser wird u.a. durch dendritische Zellen, Makrophagen und B-Lymphozyten exprimiert [72]. Hierbei kommt es nach Freisetzung von Interleukin 2 (IL-2) zur selbstinduzierten Proliferation und im Verlauf zur Ausschüttung von weiteren Zytokinen. Diese bestimmen die weitere Einteilung in einen Typ 1 (Ausschüttung u.a. von Interferon γ/IFN- γ, Tumor Nekrose Faktor α/TNF α und IL-2) bzw. Typ 2 (Ausschüttung u.a. von IL-4 und 5) [72, 74]. Eine Stimulation durch auch als T-Helferzellen bezeichnete, CD4 positive Zellen vom Typ 1

(TH1) ist essenziell für die Proliferation und Effizienz von CTLs in der Tumorabwehr. T-Helferzellen vom Typ 2 (TH2) fördern dagegen die humorale B-Zell-Antwort im lymphatischen Gewebe [75]. Zudem können CD4 positive T-Lymphozyten durch Freisetzung von IFN- γ und TNF α auch direkt zytotoxisch auf Tumorzellen wirken [75]. Den anti-tumoral wirksamen T-Lymphozyten stehen die meist Foxp3, CD25 und CD4 positiven, regulatorischen T-Lymphozyten (Tregs) gegenüber [70]. Diese fördern durch Freisetzung immunsuppressiver Zytokine wie IL10 und dem Transformierenden-Wachstumsfaktor-β (TGF-β) u.a. das Tumorwachstum und die Bildung von Metastasen [70]. Tumorzellen und Makrophagen des anti-inflammatorischen Typs 2 sezernieren Chemokine, welche Tregs rekrutieren und zu einer Infiltration dieser in das Tumorstroma führen [76].

2.4.2 Die Rolle des Immunkompartiments in der Entstehung und Progression von malignen Tumoren

Das Immunkompartiment stellt eine der am intensivsten untersuchten Komponenten von malignen Tumoren dar [77]. Die Fähigkeit, sich einer Eliminierung durch das Immunsystem zu entziehen, ist dabei ein wichtiges Merkmal in der Entstehung von Neoplasien. Seit dem Jahr 2011 wird die Immunevasion zu den *hallmarks of cancer* von Weinberg and Hanahan gezählt [78]. Die Rolle des Immunsystems in der Entstehung, Progression und Kontrolle von Tumoren ist dabei jedoch komplex: Die Zerstörung von Krebszellen durch Immunzellen als effektive anti-tumorale Reaktion steht einem pro-tumoralen Effekt der Immunzellen z.B. im Rahmen einer (chronischen) Entzündungssituation gegenüber [77, 79, 80]. Diese ambivalente Rolle wird deutlich in der Tatsache, dass einerseits immunsupprimierte Patienten mit einer zu schwachen Immunantwort zur vermehrten Bildung von Tumoren neigen, andererseits aber auch Patienten mit einer dauerhaft aktivierten Immunantwort (z.B. bei Helicobacter pylori Infektionen, Leberzirrhosen und chronischen Darmentzündungen) ein erhöhtes Malignomrisiko zeigen [49, 50, 77, 81]. Es stellt sich also die Frage, wie Tumorzellen in ihrer Interaktion mit dem Immunkompartiment sich einer Eliminierung entziehen und darüber hinaus dieses zur weiteren Progression sogar nutzen.

Eine Beschreibung des Zusammenspiels von Tumorzelle und Immunkompartiment liefert die Theorie des Immunoediting nach Schreiber et al.: Hierbei werden drei Phasen der Tumorentstehung unterschieden [80, 82]. In der initialen *Eliminationsphase* werden die Tumorzellen durch ein Zusammenspiel von

angeborenem und erworbenem Immunsystem erkannt und zerstört. Die Zellvarianten, welche diesen Prozess überleben, treten in die zweite Phase, dem sogenannten *Equilibrium* ein. Hier kontrollieren Immunzellen die malignen Zellen, schaffen es dabei jedoch nicht, sie endgültig zu zerstören. Es handelt sich um einen bis zu Jahrzehnte lang andauernden Gleichgewichtszustand, in dem der Tumor klinisch unsichtbar bleibt. Sobald die Tumorzellen die Fähigkeit erlangen, sich dieser Immunkontrolle zu entziehen, beginnt der letzte Abschnitt (*Escape-Phase*). In diesem proliferieren die Zellen unkontrolliert und werden als Tumor klinisch apparent.

Für den Übergang von Equilibrium zur Escape-Phase werden zahlreiche Mechanismen diskutiert: So entwickeln im Niveau des Tumorzellkompartiments maligne Zellen selbst eine Resistenz gegen die Immunabwehr. Dies zeigt sich u.a. in einer verminderten Expression von HLA-Antigenen auf der Zelloberfläche sowie einer reduzierten Sensibilität für zytotoxische Zytokine durch permanente Aktivierung von Transkriptionsfaktoren und anti-apoptotischen Molekülen [80, 83, 84]. Zudem trägt im Immunzellkompartiment die Rekrutierung modulierender Immunzellen (Tregs, MDSCs) und die Expression von Immuncheckpoints zum unkontrollierten Wachstum bei [80]. Im Verlauf kann sich auch die Wirkung inflammatorischer Zytokine (u.a. TNFα) verändern, welche nach einem initial protektiven Effekt später eine onkogene Wirkung entfalten können [80, 85, 86]. Außerdem haben von Tumorzellen selbst ausgeschüttete Zytokine wie TGF-β z.T. einen immunsuppressiven Effekt [87].

Das Immunkompartiment und insbesondere die geschilderten Escape-Mechanismen sind daher von großer Relevanz in der Pathogenese maligner Erkrankungen. Folgerichtig stellen sie bedeutsame Ansatzpunkte für zielgerichtete Therapiekonzepte dar [88].

2.4.3 Immuncheckpoint-Inhibitoren

Die Expression von Immuncheckpoints zählt zu den wichtigsten Escape-Mechanismen von malignen Tumoren und das Wiedererlangen einer adäquaten Immunreaktion durch deren Blockade stellt eine häufig eingesetzte Therapiestrategie dar [89]. Immuncheckpoints sind regulatorisch wirksame Membranproteine, welche sowohl pro- als auch antiinflammatorische Eigenschaften besitzen können [90, 91]. Ihre Wirkung entfalten sie typischerweise nach Bindung an entsprechende Liganden. Die Immuncheckpoints OX40, CD40 und CD137 erfüllen in der Folge beispielsweise eine immunstimulierende/proinflammatorische Funktion, wohingegen die

Immuncheckpoints Programmed Cell Death Protein-1 (PD-1), Cytotoxic T-Lymphocyte-associated Protein-4 (CTLA-4), Lymphocyte Activation Gene 3 (LAG3), V-domain Ig Suppressor of T cell Activation (VISTA) und Indolamin-2,3-Dioxygenase (IDO) zu den immuninhibierenden/antiinflammatorischen Immuncheckpoints zählen [91-93]. Aktuelle Theorien zum Wirkmechanismus fokussieren sich zumeist auf die Expression der anti-inflammatorischen Immuncheckpoints auf T-Lymphozyten und ihrer entsprechenden Liganden auf Tumorzellen bzw. anderen Zellen des TMEs [94]. Durch eine Hochregulierung und Bindung von Ligand und Rezeptor kommt es dabei zur Entstehung eines immunsuppressiven TMEs. Die phänotypisch Checkpoint exprimierenden T-Lymphozyten zeigen dabei eine verminderte Fähigkeit zur Zytokinbildung, Proliferation und Zytotoxizität [95]. Dies wird auch als Erschöpfungszustand (*engl. T Cell Exhaustion*) bezeichnet und ermöglicht den Tumorzellen, einer Eliminierung durch das Immunsystem zu entgehen [95, 96]. Darüber hinaus ist eine Expression von Checkpoints auch auf nicht lymphozytären Zellen u.a. für Tumor- und Endothelzellen beschrieben [97-99]. Die Bedeutung für die Immunmodulation ist hierbei häufig nicht eindeutig geklärt.

Immuncheckpoint-Inhibitoren (ICIs) sind therapeutisch genutzte Antikörper gegen antiinflammatorische Checkpoints oder ihre Liganden. Durch eine Blockade von deren Bindung kommt es zu einer Unterbrechung der inhibitorischen Signalwege und in der Folge zu einer verstärkten Immunantwort mit Elimination von Tumorzellen [100]. Im Jahr 2011 wurde mit dem CTLA-4-Inhibitor Ipilimumab der erste ICI überhaupt für metastasierte maligne Melanome durch die amerikanische Food and Drug Administration (FDA) zugelassen. 2014 erfolgte die Erstzulassung der PD-1 Inhibitoren Pembrolizumab und Nivolumab bei gleicher Diagnose [101, 102]. Heute kommen ICIs gegen PD-1, PD-L1 und CTLA-4 bei über 50 verschiedenen Tumorentitäten in Erst- oder Zweitlinientherapie zum Einsatz und solche gegen andere Immuncheckpoints wie LAG3 und VISTA werden im Rahmen klinischer Studien untersucht [101, 103, 104].

Während sich einerseits lang andauernde Komplettremissionen unter dem Einsatz der ICIs zeigen, profitiert in Summe nur eine Minderheit der Patienten von ihrem Gebrauch [101, 102, 105]. Aufgrund potenzieller Nebenwirkungen sowie der hohen Kosten dieser Therapiestrategien ist daher die Entwicklung prädiktiver Marker von großer Bedeutung [105, 106]. Für das Ansprechen auf eine Anti-PD-1/PD-L1-Therapie konnten in Studien bislang einige solcher Faktoren identifiziert werden: So korrelieren möglicherweise

eine hohe Mutationslast der Tumorzellen (*engl. tumor mutation burden*) und eine vermehrte Zahl an Neoantigenen (*engl. neoantigen load*) mit einem höheren Therapieansprechen [107]. Gleiches gilt für bestimmte immunhistochemisch nachweisbare Phänotypen (u.a. hohe PD-L1-Expression, Tumorinfiltration durch T-Lymphozyten) sowie im peripheren Blut detektierte erhöhte Spiegel PD-L1 tragender Tumorzellen [107, 108]. Auch individuelle Aspekte wie das Alter oder das Auftreten immunologischer Reaktionen unter Therapie können Prädiktoren für das Ansprechen auf eine Anti-PD-1/PD-L1-Therapie darstellen [107].

Die immunhistochemische Bestimmung der PD-L1 Expression hat dabei im klinischen Alltag eine große Relevanz [108]. Es existieren verschiedene Scoring-Systeme, welche die PD-L1 Expression auf Tumorzellen (Tumor Proportion Score, TPS), Immunzellen (Immune Cell Score, IC-Score) bzw. eine Kombination hiervon (Combined Positivity Score, CPS) evaluieren [109]. Die Scores werden je nach Tumorentität unterschiedlich verwendet. Bei Überschreiten eines festgelegten Cut-off Wertes indizieren sie häufig eine Therapie mit ICIs gegen PD-1 bzw. PD-L1 [109]. Nichtsdestotrotz ist die Sensitivität und Spezifität der PD-L1 Expression als prädiktiver Biomarker umstritten, da z.T. auch Patienten ohne PD-L1 Expression ein Therapieansprechen zeigen können [110, 111].

In der S3-Leitlinie für das EAC sind Therapien mit ICIs bislang nicht vorgesehen [26]. Gleichwohl untersuchen aktuell drei klinische Phase-III-Studien den Stellenwert einer Anti-PD-1-Therapie als Erstlinientherapie in fortgeschrittenen Tumorstadien. Die Veröffentlichungen erster Ergebnisse deuten dabei auf eine therapeutische Relevanz hin. In der CheckMate 649 Studie wird in einem Kollektiv von Patienten mit HER2/neu negativen Adenokarzinomen des Magens, des gastroösophagealen Übergangs (AEG-Tumore) und des Ösophagus der PD-1-Inhibitor Nivolumab in Kombination mit konventioneller Chemotherapie versus alleinige Chemotherapie getestet [40]. In einer nach CPS getrennten Subgruppenanalyse zeigt sich in der Gruppe der Patienten mit einem CPS ≥ 5 ein Überlebensvorteil sowohl im progressionsfreien Überleben als auch im Gesamtüberleben. Dies zeigt sich ebenso in der Subgruppe der Patienten mit einem CPS ≥ 1. Die ATTRACTION-4 Studie untersucht ebenfalls die Kombination Nivolumab plus Chemotherapie versus alleinige Chemotherapie bei HER2/neu negativen Patienten [112]. Hierbei werden jedoch ausschließlich Adenokarzinome des Magens sowie AEG-Tumoren berücksichtigt, ohne Evaluation des CPS und in einem

asiatischen Patientenkollektiv. Erste Ergebnisse zeigen einen Vorteil im progressionsfreien Überleben, nicht aber im Gesamtüberleben in der Gruppe mit Nivolumab. Darüber hinaus vergleicht die KEYNOTE-590 Studie die Kombination des PD-1-Inhibitors Pembrolizumab plus Chemotherapie versus alleinige Chemotherapie beim ösophagealen Plattenepithelkarzinom (ESCC), dem EAC und proximalen AEG Tumoren (Siewert I) [113]. Für Patienten mit einem CPS ≥ 10 zeigt sich in der Gesamtkohorte sowohl ein verbessertes progressionsfreies Überleben als auch ein verbessertes Gesamtüberleben. Der Großteil der Gruppe (73%) stellt jedoch ESCCs dar, wobei der Überlebensvorteil übereinstimmend auch in der kleinen EAC Subgruppe bestehen bleibt.

Eine Immuncheckpoint-Blockade der PD-1/PD-L1 Achse kann auf Grundlage dieser Ergebnisse zukünftige Therapiekonzepte beim EAC ergänzen. Darüber hinaus muss die Rolle weiterer Immuncheckpoints hingegen noch näher untersucht werden. Analog zur klinischen Praxis für PD-L1 stellt hierbei zunächst die Bestimmung ihrer genauen Verteilung eine Grundlage für prädiktive Fragestellungen in der Zukunft dar. Für die Immuncheckpoints VISTA, LAG3 und IDO wurde im Rahmen dieser Arbeit durch immunhistochemische und RNA-Sonden (RNA BaseScope) basierte Verfahren erstmals deren Expression in einem großen Kollektiv von Patienten mit EAC untersucht und ihre Bedeutung durch Korrelation mit klinischen Daten analysiert.

2.4.4 Dynamik des Immunkompartiments unter neoadjuvanter Therapie und Metastasierung

Das Immunkompartiment spielt eine entscheidende Rolle in der Tumorkontrolle und seine Zusammensetzung kann Hinweise für das zu erwartende Ansprechen auf eine systemische Therapie liefern [77, 114, 115]. Während neoadjuvante Therapiekonzepte ein wichtiger Bestandteil in der kurativen Behandlung zahlreicher Tumore sind, bleiben ihre Auswirkungen auf das Immunkompartiment häufig unklar. Dabei ist die Studienlange auch für das EAC extrem limitiert [116]. Kelly et al. vergleichen in einem Kollektiv von 31 Patienten die Expression von Immuncheckpoints in prä- und postneoadjuvant gewonnenen Gewebeproben mittels Immunhistochemie und quantitativer-Echtzeit-Polymerase-Kettenreaktion (qPCR) [116]. Hierbei zeigt sich eine Hochregulation sowohl immunstimulierender als auch -inhibierender Immuncheckpoints wie LAG3, PD-L1, OX40 und CD137 und eine verstärkte Tumorinfiltration durch CD8 positive T Lymphozyten nach erfolgter Neoadjuvanz. Eine

andere Studie von van der Kraak et al. weist darüber hinaus eine vermehrte PD-L1 Expression in ösophagealen Zelllinien durch Zugabe von 5-Fluorouracil nach, kann dieses aber in vivo bei EAC Patienten nicht bestätigen [117].

In anderen Tumorentitäten zeigen sich kontroverse Ergebnisse bzgl. des Einflusses neoadjuvanter Therapien auf das Immunkompartiment [118]. So ergeben Studien zu Mammakarzinomen sowie nicht-kleinzelligen-Bronchialkarzinomen eine vermehrte Infiltration von Immunzellen sowie eine verstärkte Expression von Immuncheckpoints in Folge einer neoadjuvanten Chemotherapie [119, 120]. Dagegen zeigen sich reduzierte Mengen zirkulierender bzw. tumorinfiltrierender Immunzellen für Zervix- und Kolonkarzinome [121, 122]. Ein Ansprechen auf ICIs scheint jedoch mit der Anzahl an Tumor infiltrierenden Lymphozyten (TILs) und einem inflammatorischen TME zu korrelieren [114, 119]. Daher könnten Erkenntnisse über den Einfluss einer neoadjuvanten Therapie auf das Immunkompartiment dazu beitragen, einen möglicherweise synergistischen Effekt der Kombination von ICIs mit einer (Radio-) Chemotherapie zu evaluieren.

Daneben existieren bislang ebenso keine vergleichenden Studien zur Zusammensetzung des Immunkompartiments zwischen unbehandeltem Primärtumor und hämatogenen Metastasen beim EAC. Dabei ist anzunehmen, dass auch die lokal unterschiedlichen Bedingungen in Kombination mit einer neoadjuvanten Therapie die Zusammensetzung des TMEs in den Metastasen beeinflussen. So konnte für das Mammakarzinom beispielsweise eine verminderte Zahl von TILs sowie eine geringere Expression von PD-L1 und MHC I in metastatischen Läsionen gegenüber dem Primärtumor gezeigt werden [123]. Beim Rektumkarzinom ist darüber hinaus ein unterschiedliches Ansprechen auf eine neoadjuvante Chemotherapie von Primärtumor und korrespondierenden Lebermetastasen beschrieben [124, 125].

Über die therapieabhängigen Veränderungen des Immunkompartiments beim EAC ist somit insgesamt wenig bekannt. Daher wurden im Rahmen dieser Arbeit entweder primär operierte oder therapienaive, endoskopisch gewonnene Tumorproben mit neoadjuvant vorbehandeltem Tumor des Ösophagus und seinen hämatogenen Metastasen verglichen. Die Genexpression wurde mittels der NanoString® Technologie auf RNA-Ebene analysiert und Expressionsunterschiede herausgearbeitet. Neben den unten aufgeführten, publizierten Ergebnissen befindet sich ein Teil dieser Daten derzeit im Review („*Gene expression changes in metastatic*

esophageal adenocarcinoma“ von Wagener, Kraemer et al., submitted to Cancers, Basel) und wird in Zukunft dabei helfen, das Immunkompartiment der hämatogenen EAC Metastasen näher zu charakterisieren. Im Diskussionsteil dieser Arbeit soll daher auch kurz Bezug auf die vorläufigen Ergebnisse dieses Projekts genommen werden.

2.5. Biomarker in ErbB-Rezeptor abhängigen Signalwegen im Tumorzellkompartiment

2.5.1 ErbB-Rezeptoren und ihre Signalwege

Die ErbB-Rezeptor-Familie umfasst die vier transmembranären Tyrosinkinase-Rezeptoren EGFR (HER1), HER2, HER3 und HER4 [126]. Sie werden ubiquitär in epithelialem, mesenchymalem und neuronalem Gewebe exprimiert und haben eine große Bedeutung für die Proliferation, Differenzierung und Migration der Zellen [127]. Liganden stellen u.a. EGF, TGF-α, heparin binding EGF (HB-EGF), Amphiregulin, Betacellulin, Neuregulin und Epiregulin dar [128]. Dabei existieren z.T. unterschiedliche Präferenzen für die einzelnen Subtypen der Rezeptoren [128]. Die genauen Mechanismen der Signaltransduktion und Interaktion der einzelnen Signalwege sind komplex und Gegenstand aktueller Forschung [129]. Grundsätzlich kommt es nach Bindung eines Liganden jedoch zur Dimerisierung des Rezeptors mit einem anderen Mitglied der ErbB-Rezeptor-Familie [130]. Anschließend wird am intrazellulären Ende des Rezeptors ein Tyrosinrest phosphoryliert und in der Folge werden unterschiedliche Signalkaskaden aktiviert [130]. Drei Schlüsselsignalwege stellen hierbei der Ras/Raf/MEK/ERK- (MAP3K-Weg), der PI3K/PTEN/Akt/mTOR- sowie der JAK/STAT-Signalweg dar (s. Abbildung 4) [131, 132].

In der Endstrecke kommt es zur Aktivierung von Transkriptionsfaktoren im Zellkern, welche zur u.a. Initiierung von Mitose, Zellwachstum, Differenzierung, Migration und Apoptoseresistenz führen [127, 133]. ErbB-Rezeptor abhängige Signalwege spielen

daher sowohl unter physiologischen Bedingungen als auch in der Entstehung einer Vielzahl solider Tumoren eine wichtige Rolle [134].

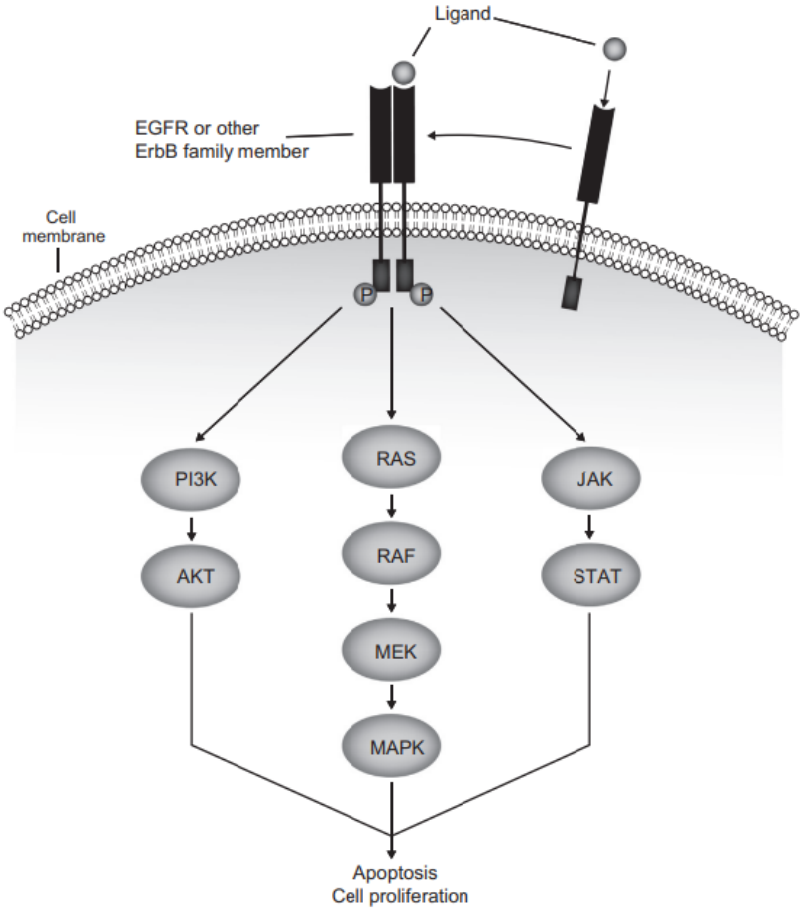

Abbildung 4: (Roengvoraphoj et al., Cancer Treatment Reviews, 2013 [132])
Vereinfachte Darstellung ErbB-Rezeptor abhängiger Signalwege

2.5.2 Ausgewählte Amplifikationen in ErbB-Rezeptor abhängigen Signalwegen und ihre klinische Relevanz

Im Tumorzellkompartiment können Alterationen der ErbB-Rezeptor abhängigen Signalwege auf unterschiedlichen Ebenen stattfinden [135]. Oft stellen sie dabei entscheidende Treiber in der Entstehung von malignen Erkrankungen dar [135]. Sowohl die Funktion der Rezeptoren als auch die der nachgeschalteten intrazellulären Proteine können durch Mutationen, Überexpression und Veränderungen der Genkopiezahl (*engl. copy number variation, CNV)* dysreguliert sein [136, 137].

Das Auftreten von CNVs ist auch in der Entstehung des EACs von großer Bedeutung [138]. TCGA Daten von 87 EAC Patienten zeigen u.a. Amplifikationen von HER2/neu in 28,7%, von KRAS in 9,2% und von PIK3CA in 4,6% [139, 140]. Die klinische

Relevanz wird deutlich in der Etablierung des HER2/neu Antikörpers Trastuzumab bei HER2/neu amplifizierten EACs [26]. Darüber hinaus stellen KRAS Amplifikationen bzw. Mutationen einen bekannten Resistenzmechanismus für zielgerichtete Therapien gegen EGFR in anderen Tumorentitäten dar [141, 142]. Neuere Studien zu KRAS amplifizierten Magenkarzinomen diskutieren zudem zielgerichtete Therapiemöglichkeiten im MAP3K-Signalweg, wie den Einsatz einer dualen MEK und SHP2 Blockade [143]. PIK3CA Inhibitoren wurden erstmals 2014 als Drittlinientherapie bei therapierefraktären chronisch lymphatischen Leukämien eingesetzt [144]. Für gastrointestinale Tumoren kann bisher lediglich in Zellkulturen der positive Effekt eines solchen Inhibitors belegt werden [145]. Klinische Studien zeigen dagegen keinen signifikanten Überlebensvorteil dieser Therapie beim Magenkarzinom [146].

Amplifikationen in ErbB-Rezeptor abhängigen Signalwegen des Tumorzellkompartiments können, wie das Beispiel HER2/neu gezeigt hat, weitreichende Konsequenzen für den klinischen Alltag haben. Studien zu diesen Biomarkern beim EAC sind bislang selten: Ihre Häufigkeit, der prognostische Wert und etwaige Auswirkungen auf andere Tumorkomponenten müssen daher weiter evaluiert werden [147-149]. Im Rahmen dieser Arbeit wurde das Auftreten von PIK3CA, KRAS und HER2/neu Amplifikationen beim EAC mittels FISH-Analytik untersucht und eine entsprechende Proteinexpression durch immunhistochemische Verfahren dargestellt. Die Ergebnisse wurden durch Korrelation mit Biomarkern des Immunkompartiments und klinischen Daten weiter ergänzt.

3. Zielsetzung der Arbeit

Das übergeordnete Thema dieser Arbeit war die Identifizierung prädiktiver und prognostischer Biomarker in unterschiedlichen Tumorkompartimenten des EACs. Dabei wurde ein Verfahren zur Identifizierung Kompartiment-spezifischer Biomarker als Alternative zum Single Cell Sequencing entwickelt. Zudem wurden konkrete Biomarker im EAC Gewebe (u.a. Immuncheckpoint-Proteine, Genamplifikationen in ErbB-Rezeptor abhängigen Signalwegen) durch unterschiedliche Methoden analysiert und auf ihre klinische Bedeutung hin überprüft. Entsprechend der drei Schwerpunktthemen ergaben sich folgende Fragestelllungen und Ziele:

1. Entwicklung einer Methode zur Identifizierung Kompartiment-spezifischer Biomarker

- Etablierung und Validierung eines alternativen Verfahrens zum Single Cell Sequencing für die Kompartiment-spezifische Transkriptomanalyse des mesenchymalen-, epithelialen- und Immunkompartiments
- Optimierung des Verfahrens für möglichst kleine Zellmengen zur Anwendung an endoskopisch gewonnenen Gewebeproben
- Exemplarische Beschreibung von differenziellen Genexpressionen zwischen gesunder Ösophagus-Mukosa und EAC-Gewebe

2. Biomarker des Immunkompartiments und deren Dynamik unter Metastasierung und Chemotherapie

- Wie häufig finden sich die immunsuppressiv wirksamen Immuncheckpoint-Proteine VISTA, LAG-3 und IDO beim EAC?
- Welche Verteilung zeigt ihre Expression auf Tumorzellen und Entzündungszellen im Tumorstroma?
- Gibt es Korrelationen mit anderen Immunmarkern (z.B. T-Zell Marker, RNA Expression von IFNγ)?
- Lässt sich eine prognostische Relevanz durch Korrelation mit klinischen Daten für das EAC zeigen?
- Wie verändert eine neoadjuvante Therapie die Genexpression von immunologische Biomarkern im Primärtumor des EACs?

- Gibt es Hinweise für Genexpressionsunterschiede in Bezug auf immunologische Biomarker zwischen therapienaivem Primärtumor und vorbehandelten Metastasen?

3. Biomarker in ErbB-Rezeptor abhängigen Signalwegen im Tumorzellkompartiment

- Wie häufig finden sich Amplifikationen von KRAS, PIK3CA und HER2/neu in Tumorzellen des EAC?
- Gibt es Korrelationen mit anderen Biomarkern des TME?
- Lassen sich prognostische Subgruppen durch Korrelation mit klinischen Daten für das EAC zeigen?

4. Material und Methoden

Dieser Teil der Dissertation wird durch die unter Kapitel 6 genannten sieben Publikationen ersetzt.

5. Ergebnisse

Dieser Teil der Dissertation wird durch die unter Kapitel 6 genannten sieben Publikationen ersetzt.

6. In der Dissertation diskutierte Publikationen

Erstautorenschaften

Cell type-specific transcriptomics of esophageal adenocarcinoma as a scalable alternative for single cell transcriptomics
Krämer M*, Plum PS*, Velazquez Camacho O, Folz-Donahue K, Thelen M, Garcia-Marquez I, Wölwer C, Büsker S, Wittig J, Franitza M, Altmüller J, Löser H, Schlößer H, Büttner R, Schröder W, Bruns CJ, Alakus H, Quaas A, Chon SH, Hillmer AM.
Molecular Oncology 2020 Jun;14(6):1170-1184. (Impact Factor: 6,574)
doi: 10.1002/1878-0261.12680. Epub 2020 Apr 21. PMID: 32255255; PMCID: PMC7266280.
*geteilte Erstautorenschaft

The expression of the immune checkpoint regulator VISTA correlates with improved overall survival in pT1/2 tumor stages in esophageal adenocarcinoma
Heike Loeser*, **Max Kraemer***, Florian Gebauer, Christiane Bruns, Wolfgang Schröder, Thomas Zander, Oana-Diana Persa, Hakan Alakus, Arnulf Hoelscher, Reinhard Buettner, Philipp Lohneis and Alexander Quaas
Oncoimmunology. 2019 Feb 27;8(5):e1581546. (Impact Factor: 5,869)
doi: 10.1080/2162402X.2019.1581546. PMID: 31069143; PMCID: PMC6492979.
*geteilte Erstautorenschaft

Lymphocyte activation gene-3 (LAG3) mRNA and protein expression on tumour infiltrating lymphocytes (TILs) in oesophageal adenocarcinoma.
Gebauer F*, **Krämer M***, Bruns C, Schlößer HA, Thelen M, Lohneis P, Schröder W, Zander T, Alakus H, Buettner R, Loeser H, Quaas A.
Journal of Cancer Research and Clinical Oncolology 2020 Sep;146(9):2319-2327. (Impact Factor: 3,656)
doi: 10.1007/s00432-020-03295-7. Epub 2020 Jun 26. PMID: 32592066; PMCID: PMC7382658.
*geteilte Erstautorenschaft

Indoleamine 2.3-dioxygenase (IDO) expression is an independent prognostic marker in esophageal adenocarcinoma
Heike Loeser*, **Max Kraemer***, Florian Gebauer, Christiane Bruns, Wolfgang Schröder, Thomas Zander, Hakan Alakus, Arnulf Hoelscher, Reinhard Buettner, Philipp Lohneis, Alexander Quaas
Journal of Immunology Research, Volume 2020, Article ID 2862647 (Impact Factor: 3,327),
doi: 10.1155/2020/2862647
*geteilte Erstautorenschaft

Co-Autorenschaften:

PIK3CA and KRAS Amplification in Esophageal Adenocarcinoma and their Impact on the Inflammatory Tumor Microenvironment and Prognosis
Essakly A, Loeser H, **Kraemer M**, Alakus H, Chon SH, Zander T, Buettner R, Hillmer AM, Bruns CJ, Schroeder W, Gebauer F, Quaas A.
Translational Oncology. 2020 Feb;13(2):157-164. (Impact Factor: 3,558)
doi: 10.1016/j.tranon.2019.10.013. Epub 2019 Dec 19. PMID: 31865178; PMCID: PMC6931191.

HER2/neu (ERBB2) expression and gene amplification correlates with better survival in esophageal adenocarcinoma.
Plum PS, Gebauer F, **Krämer M**, Alakus H, Berlth F, Chon SH, Schiffmann L, Zander T, Büttner R, Hölscher AH, Bruns CJ, Quaas A, Loeser H.
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Wagener-Ryczek S, Schoemmel M, **Kraemer M**, Bruns C, Schroeder W, Zander T, Gebauer F, Alakus H, Merkelbach-Bruse S, Buettner R, Loeser H, Thelen M, Schlößer HA Quaas A.
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doi: 10.1007/s00262-019-02475-w. Epub 2020 Jan 20. PMID: 31960110; PMCID: PMC7113210.

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Cell type-specific transcriptomics of esophageal adenocarcinoma as a scalable alternative for single cell transcriptomics

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Keywords
cancer-associated fibroblasts; cell types; esophageal adenocarcinoma; transcriptomics; tumor microenvironment

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Single-cell transcriptomics have revolutionized our understanding of the cell composition of tumors and allowed us to identify new subtypes of cells. Despite rapid technological advancements, single-cell analysis remains resource-intense hampering the scalability that is required to profile a sufficient number of samples for clinical associations. Therefore, more scalable approaches are needed to understand the contribution of individual cell types to the development and treatment response of solid tumors such as esophageal adenocarcinoma where comprehensive genomic studies have only led to a small number of targeted therapies. Due to the limited treatment options and late diagnosis, esophageal adenocarcinoma has a poor prognosis. Understanding the interaction between and dysfunction of individual cell populations provides an opportunity for the development of new interventions. In an attempt to address the technological and clinical needs, we developed a protocol for the separation of esophageal carcinoma tissue into leukocytes (CD45+), epithelial cells (EpCAM+), and fibroblasts (two out of PDGFRα, CD90, anti-fibroblast) by fluorescence-activated cell sorting and subsequent RNA sequencing. We confirm successful separation of the three cell populations by mapping their transcriptomic profiles to reference cell lineage expression data. Gene-level analysis further supports the isolation of individual cell populations with high expression of *CD3*, *CD4*, *CD8*, *CD19*, and *CD20* for leukocytes, *CDH1* and *MUC1* for epithelial cells, and *FAP*, *SMA*, *COL1A1*, and *COL3A1* for fibroblasts.

Abbreviations
CAF, cancer-associated fibroblast; DEG, differentially expressed gene; DMSO, dimethyl sulfoxide; EAC, esophageal adenocarcinoma; EMT, epithelial-to-mesenchymal transition; FACS, fluorescence-activated cell sorting; FAP, fibroblast activation protein; FBS, fetal bovine serum; FDR, false discovery rate; FITC, fluorescein isothiocyanate; GO, gene ontology; LD, life dead; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; PCA, principal component analysis; PDGF, platelet-derived growth factor; PDGFRα, PDGF receptor α; PD-L1, programmed death ligand 1; RCA, reference component analysis; scRNA-seq, single-cell RNA sequencing; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

As a proof of concept, we profiled tumor samples of nine patients and explored expression differences in the three cell populations between tumor and normal tissue. Interestingly, we found that angiogenesis-related genes were upregulated in fibroblasts isolated from tumors compared with normal tissue. Overall, we suggest our protocol as a complementary and more scalable approach compared with single-cell RNA sequencing to investigate associations between clinical parameters and transcriptomic alterations of specific cell populations in esophageal adenocarcinoma.

1. Introduction

Esophageal cancer is associated with the sixth-highest mortality rate of cancer-related deaths and presumed to be one of the most mortal malignancies worldwide (Coleman *et al.*, 2018). Over the past decades, there has been a rapid increase particularly in the incidence of esophageal adenocarcinoma (EAC) in the Western world, but despite improvements in perioperative treatments, there is no sufficient therapeutic strategy for the majority of patients so far (Coleman *et al.*, 2018). Further, prediction of therapy success is poor and response to neoadjuvant therapies varies dramatically ranging from no to complete response (Vallböhmer *et al.*, 2010).

Focus in cancer research has shifted from considering epithelial cancer cells to analyses of their interactions with different components of the tumor microenvironment. Particularly, the immune cell population has revealed an immense effect on tumor progression. Today, immune checkpoint blockage is first-line therapy in several solid neoplasia such as malignant melanoma and non-small cell lung cancer (NSCLC) (Incorvaia *et al.*, 2019; Spagnolo *et al.*, 2019). For EAC, several biomarkers in the immune compartment have been detected, and currently, clinical trials are ongoing to evaluate the safety and efficacy of possible immunotherapies (Tanaka *et al.*, 2017).

There is also rising evidence for an important impact of cancer-associated fibroblasts (CAFs) on tumor biology and disease progression (Shiga *et al.*, 2015). CAFs are a heterogeneous cell population of unknown origin that form the stromal part of solid tumors (Shiga *et al.*, 2015). They release cytokines (e.g., TGF-β), proteases (e.g., matrix metalloproteinases), and growth factors (e.g., VEGF, PDGF) with various autocrine and paracrine functions that can enhance tumor growth, neovascularization, and migration of cancer cells (Kakarla *et al.*, 2012). In several cancer types, including colorectal, breast, ovarian, and head and neck cancer, the presence of CAFs correlates with poor prognosis (Lai *et al.*, 2012; Marsh *et al.*, 2011; Tsujino *et al.*, 2007; Yamashita *et al.*, 2012). In esophageal cancer, Wang et al. described pleiotropic functions in carcinogenesis, proliferation, angiogenesis, and metastasis (Wang *et al.*, 2016). Although CAFs obviously influence tumor biology, they are still poorly characterized. Since targeting either CAFs or their secreted paracrine factors could improve therapeutic response, characterization of the roles of fibroblasts in EAC can help developing clinically effective treatments (Kakarla *et al.*, 2012).

Single-cell transcriptome sequencing (scRNA-seq) has opened new avenues for the understanding of the biological role of cell populations, their origins, and interactions (Ren *et al.*, 2018). Single-cell approaches – in contrast to bulk tissue sequencing – allow to determine which cell type is responsible for transcriptomic changes, which is of fundamental importance for the understanding of tumor biology. Single-cell RNA sequencing of colorectal cancers, for example, has shown that epithelial-to-mesenchymal transition (EMT) signature genes are upregulated in CAFs and not in epithelial cells (Li *et al.*, 2017), a phenomenon that might have gotten misinterpreted as EMT by bulk sequencing. Hence, a systematic analysis of gene expression profiles in different tumor cell types of EAC in comparison with normal esophageal mucosa will help to understand the role of the interaction between (cancer associated) fibroblasts, immune cells, and epithelial tumor cells in carcinogenesis and disease progression. Single-cell sequencing, however, is still expensive, limiting the number of clinical samples that are usually profiled per study. More scalable approaches for the analysis of individual cell populations of larger series of tumors are warranted to be able to identify cell type-specific alterations that are associated with clinical features. It is therefore desirable to develop economic methodologies that allow to analyze cell types separately but not necessarily on a single-cell level.

To address this need, we developed a protocol to isolate different cell types (epithelial cells, immune cells, fibroblasts) of endoscopically obtained EAC tissue as well as normal esophageal mucosa and to sequence the transcriptome of each cell type separately. By mapping the transcriptomes to reference tissue expression data, we demonstrate the successful separation of the three cell types. Further, we explored the utility of this approach by comparing expression profiles of esophageal normal mucosa with adenocarcinoma tissues. Our protocol provides a scalable tool for the systematic and cost-effective investigation of the individual cell populations of EAC. It will help to improve our understanding of pathological processes and possibly identify novel therapeutic targets in EAC.

2. Methods

2.1. Patients and tumor samples

Fresh tissue samples from nine patients with histologically confirmed adenocarcinoma of the distal esophagus or the gastroesophageal junction were prospectively collected between June and December 2018. In six of these patients, both corresponding tumor and normal esophageal mucosa were taken, while in one patient only tumor tissue and in two patients normal mucosa had been biopsied. Processing and consecutive separation of these samples led to 31 cell populations after FACS sorting with sufficient yield for further analysis via RNA-seq.

All patients underwent primary staging including esophagogastroduodenoscopy, endoscopic ultrasound, and spiral contrast-enhanced computer tomography of thorax and abdomen within the Department of General, Visceral and Cancer Surgery at the University Hospital of Cologne. Written informed consent was obtained from all patients before participating in the analysis, and the study was approved by the local Institutional Review Board (Ethics No. 18-274). The study's methodologies conformed to the standards set by the Declaration of Helsinki and its later amendments.

Samples were taken either by endoscopic biopsy (six samples) or obtained from surgical specimens (three samples). During endoscopy, standardized biopsies from both tumor and corresponding normal esophageal mucosa at 5-cm distance were taken and immediately transferred into 1 mL of RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for further processing.

Standard surgical procedure was laparotomic or laparoscopic gastrolysis and right transthoracic en bloc esophagectomy including two-field lymphadenectomy of mediastinal and abdominal lymph nodes (Ivor Lewis esophagectomy) as described previously (Plum *et al.*, 2018). Samples obtained from a surgical specimen were also immediately transferred into RPMI 1640 medium at room temperature and further processed as described below.

In case of neoadjuvant treatment (five patients), either neoadjuvant chemoradiation analog CROSS (four patients) or perioperative chemotherapy analog FLOT regimen (one patient) was applied (Al-Batran *et al.*, 2016; van Hagen *et al.*, 2012).

2.2. Single-cell dissection

Immediately after endoscopy, corresponding tumor and normal mucosa biopsies were processed separately (Fig. 1). Tissue samples were transferred together with 1 mL of Gibco™ RPMI 1640 Medium (Thermo Fisher Scientific) and 1 mL of PBS (Thermo Fisher Scientific) into Petri dishes and dissected mechanically using two scalpels. After transfer into gentleMACS C Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany), additional 1 mL of each of the following enzymes was added: DNAse I (500 $U \cdot mL^{-1}$; AppliChem PanReac, Darmstadt, Germany; in PBS), collagenase IV (320 $U \cdot mL^{-1}$; Thermo Fisher Scientific; in PBS), and dispase II (2 $U \cdot mL^{-1}$; Sigma-Aldrich, St. Louis, MO, USA; in PBS). Automated tissue dissociation was performed using the preset human tumor programs 1, 2, and 3 of the gentleMACS™ Dissociator (Miltenyi Biotec). Mechanical dissociation steps 1 and 2 were followed by enzymatic digestion for 20 min while rotating at 37 °C. Following enzymatic-mechanical tissue dissociation, samples were diluted with PBS and filtered through a Falcon® 100-µm cell strainer (Corning, New York, NY, USA) to remove larger debris. Filtering was repeated if necessary. Dissociated cells were collected by centrifugation at 405 ***g*** for 5 min at room temperature. Cells were resuspended by vortexing within freezing medium including 60% Gibco™ RPMI 1640 medium (Thermo Fisher Scientific), 30% FBS (Capricorn Scientific, Ebsdorfergrund, Germany), and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Afterward, cells were frozen at −80 °C for 24 h and then transferred to liquid nitrogen for long-term storage until fluorescence-activated cell sorting (FACS). As one freeze/thaw cycle of the single-cell solutions resulted in hemolysis, no additional erythrocyte lysis was performed.

2.3. Immunofluorescence staining of single-cell suspension

Frozen single-cell suspensions were thawed, added to 10 mL of Gibco™ RPMI 1640 Medium (Thermo Fisher Scientific) at 37 °C and vortexed. Before further processing, 500 µL of each sample was separated and mixed with 1 mL of medium for the unstained control. Samples were centrifuged at 450 ***g*** for 5 min at room temperature. After centrifugation, all supernatants were discarded. The collected cells were resuspended in 500 µL MACS buffer [PBS (pH 7.2) + 2 mM EDTA + 0.5% BSA] and kept on ice until FACS analysis. The following incubation steps were performed on ice in the dark. Samples were stained consecutively with the following monoclonal anti-human antibodies: 2 µL Alexa Fluor® 647-conjugated anti-PDGF receptor α (PDGFRα; Cell Signaling Technology, Danvers, MA, USA) and 1 µL eBioscience™ Fixable Viability Dye eFluor™ 506 (Thermo Fisher Scientific) for 15 min followed by 5 min of incubation with 1 µL PE/Cy7-conjugated anti-CD45 (Biolegend, San Diego, CA, USA). Cells were incubated for 10 min with additional 2 µL FITC-conjugated anti-EpCAM (Miltenyi Biotec), 5 µL PE-conjugated anti-fibroblast (Miltenyi Biotec), and 2 µL VioBlue-conjugated anti-CD90 (Miltenyi Biotec). Additional staining for epithelial cells utilizing 1 µL APC/Fire™ 750-conjugated anti-mouse/human CD324 (E-Cadherin) (Biolegend) was performed in six samples. This additional staining was omitted in subsequent samples as E-Cadherin did not stain additional epithelial cells that were not stained by EpCAM, including normal esophagus (data not shown). Cells were spun down at 450 ***g*** for 5 min at 4 °C. Supernatants were discarded and collected cells resuspended in 500 µL cold MACS buffer.

Simultaneously to the cells, compensation beads were prepared for analysis by flow cytometry utilizing the ArC™ Amine Reactive Compensation Bead Kit for life-dead (LD) staining (Thermo Fisher Scientific) and the AbC™ Total Antibody Compensation Bead Kit (Thermo Fisher Scientific), respectively, according to the manufacturer's instructions. Immunofluorescent stained cell suspensions and beads were kept on ice until sorting.

2.4. Flow cytometry analysis and sorting

Sorting of the single-cell suspensions was performed using a BD FACSAria Fusion (BD Biosciences, San Jose, CA, USA) using a 100-µm nozzle and 20 psi pressure, using aerosol containment. Immediately before analysis, cell suspensions were filtered once again using a 70-µm CellTrics strainer (Sysmex, Kobe, Japan). Gating strategy was as follows: After viability gating, cells were gated according to the surface expression of CD45 as marker for immune cells ('immune cell population'). CD45-negative cells were analyzed for the expression of PDGFRα, fibroblast marker, and CD90. Those cells which were positive for at least two of those markers were defined as fibroblasts ('fibroblast cell population'). Finally, all other CD45-negative cells were analyzed for expression of EpCAM (or E-Cadherin) as marker for tumor cells of epithelial origin ('epithelial cell population'). Cell subpopulations were sorted into 500 µL cold MACS buffer at 4 °C.

2.5. RNA isolation and next-generation sequencing

After sorting, cells were kept on ice and RNA isolation was performed using the PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Isolated RNA was stored at −80 °C. Libraries for RNA sequencing were prepared using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH, Vienna, Austria) according to the low-input protocol. Libraries were sequenced on a HiSeq 4000 (Illumina) by 1× 50 bases.

2.6. RNA-seq analysis

Reads were aligned to the human genome (*Homo sapiens* GRCh38) using STAR software v. 2.6 (Dobin *et al.*, 2013). Mapped reads were counted with HTSeq, and differential gene expression analysis was conducted using BIOCONDUCTOR R package DESEQ2 version 1.22.2. (Love *et al.*, 2014). An adjusted *P*-value threshold of 0.05 and a log2 fold change ≥ 1 were set to determine differential gene expression. The complete lists of DEGs in a pairwise manner are available in the Table S1.

2.7. Reference component analysis

Clustering of the independent samples was performed using the R package reference component analysis (RCA) v. 1.0. (Li *et al.*, 2017) with the default option 'Global Panel', and this panel contains a set of featured genes from the reference bulk transcriptomes in the HumanU133A/GNF1H Gene Atlas and the Primary Cell Atlas. The resulting RCA clusters for the different samples were plotted as heat maps or principal component analysis (PCA) as part of the downstream analysis pipeline of the RCA package.

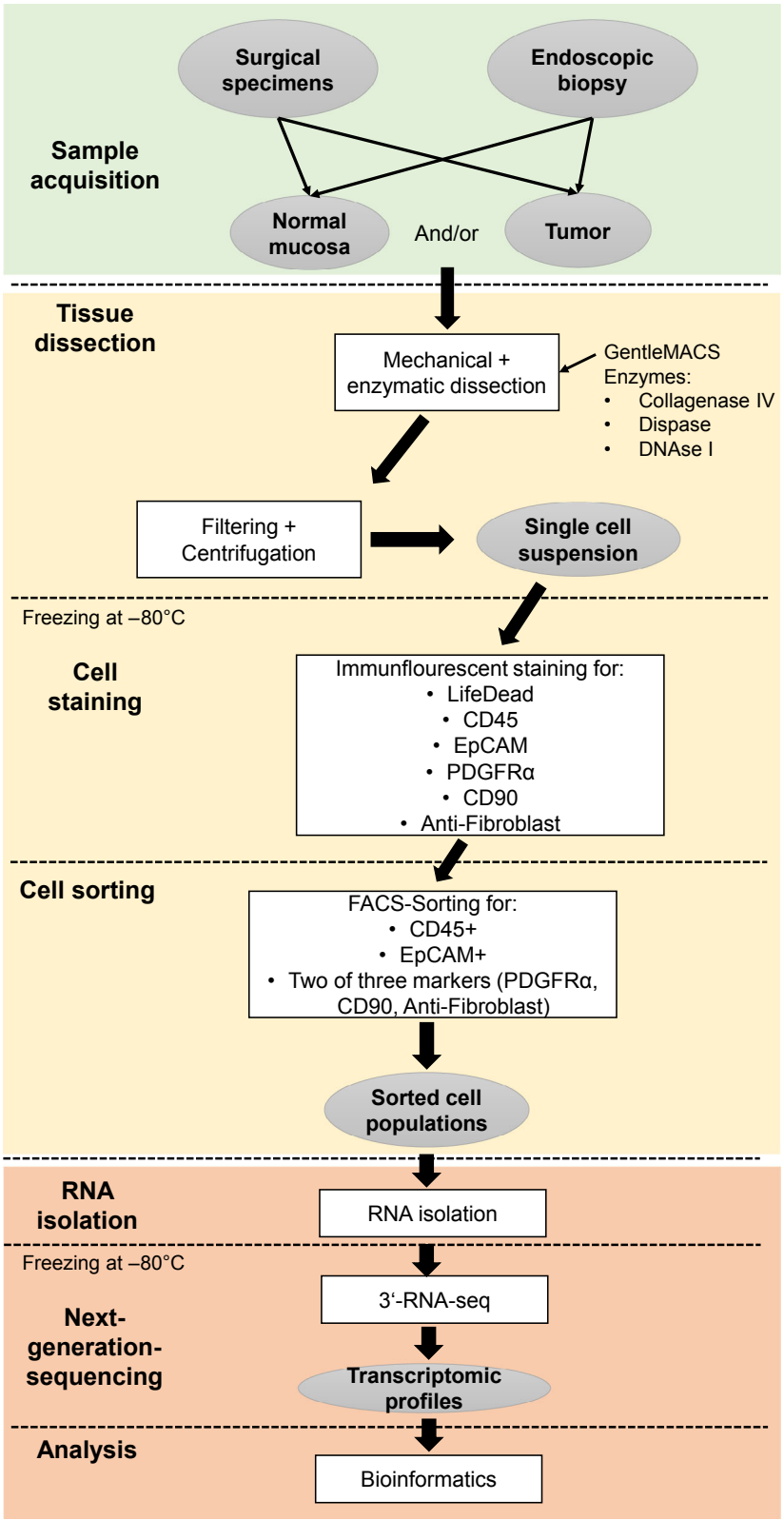

Fig. 1. Schematic representation of the workflow.

2.8. Pseudo-bulk analysis

Processed RNA-seq gene counts for all the analyzed cells were obtained from the supplementary datasets in Owen *et al.* (2018). Single-cell datasets from four patients were selected (A, D, E, and F), where A and D are patients with Barrett's esophagus and E and F are normal esophageal mucosae of patients without Barrett's esophagus. For each of the four analyzed patients, a sample of 100 single cells was randomly selected and the processed counts from each patient were aggregated by obtaining the mean for each gene across the 100 sampled cells to emulate a pseudo-bulk dataset. The resulting averaged counts were used as input to perform the RCA.

2.9. Gene ontology

After differential gene expression analysis was performed among the different flow cytometry-sorted cell populations, genes whose expression was exclusively upregulated in comparison with the remaining cell types were analyzed with the GO software (http://geneontology.org/; Ashburner *et al.*, 2000; Carbon *et al.*, 2019) to test for over-representation of common biological processes using all genes as background. To investigate tumor/normal differential expression, all expressed genes of the respective cell type defined by the sum of normalized expression tag counts > 1 were used as background.

3. Results

3.1. Patients' characteristics

Tumor samples from nine patients were collected including seven male and two female patients. Median age of all patients participating was 65 years (minimum: 57 years, maximum: 83 years). Initial tumor grading was G2 in five patients and G3 in two patients, pT stage was pT1 in one patient, pT2 in three patients, pT3 in two patients, and pT4 in one patient (no information available for 2 patients since those patients did not undergo surgery due to metastasis). Four patients received neoadjuvant chemoradiation analog CROSS, and one patient received perioperative chemotherapy analog FLOT regimen. Full baseline characteristics are provided in Table 1.

Table 1. Baseline characteristics of patient included.

	Total (n = 9)	
Variable	Number (n)	Percentage
Age		
Median (min–max)	65 years (57–83 years)	
Gender		
Male	7	77.8
Female	2	22.2
Anatomical localization		
Esophagogastric junction	8	88.9
Gastric	1	11.1
Sample origin		
Endoscopic biopsy	6	66.7
Surgical specimen	3	33.3
Samples		
Tumor	1	11.1
Normal mucosa	2	22.2
Tumor and normal mucosa	6	66.7
Kind of neoadjuvant therapy		
None	4	44.4
Neoadjuvant chemoradiation	4	44.4
Perioperative chemotherapy	1	11.1
pT category		
pT1	1	11.1
pT2	3	33.3
PT3	2	22.2
pT4	1	11.1
No information[a]	2	22.2
pN category		
pN0	3	33.3
pN1	2	22.2
pN2	0	0
pN3	2	22.2
No information[a]	2	22.2
Grading		
G1	0	0
G2	5	55.6
G3	2	22.2
No information	2	22.2

[a]No further information available since one patient had progression after primary staging and lost to follow-up in another case.

3.2. Workflow

We developed a protocol for isolating the different cell populations (immune cells, fibroblasts, epithelial cells) from EAC and corresponding esophageal mucosa to sequence the transcriptome separately for each cell type. In brief, the workflow consists of a gentle single-cell dissection, immunofluorescence staining, consecutive flow cytometry sorting, and RNA analysis of each cell fraction (Fig. 1).

Single-cell suspension was obtained simultaneously by enzymatic dissociation using DNAse I, collagenase IV, and dispase II and simultaneous mechanical dissociation with a gentleMACS™ Dissociator. Antibody

staining and subsequent sorting of the single-cell suspension were implemented on literature-based antigen selection (see below). An example of flow cytometry sorting strategy is shown in Fig. 2.

For sorting the immune cell population, we selected membrane antigen CD45, a glycoprotein which is expressed on nearly all hematopoietic cells except for mature erythrocytes and platelets (Nakano *et al.*, 1990). CD45 has been revealed as a potent marker to differentiate hematopoietic cells from carcinoma cells in solid and fluid tumor tissue via flow cytometry (Acosta *et al.*, 2016) and serves as a pan-leukocyte marker (Ruffell *et al.*, 2012), therefore ideal for detection of immune cells.

Epithelial cells were sorted for epithelial cell adhesion molecule (EpCAM), which is exclusively displayed in epithelia and epithelial-derived neoplasms (Patriarca *et al.*, 2012) and expressed in various carcinomas, including EAC (Sun *et al.*, 2018). EpCAM antibodies are commonly used to detect epithelial cells in flow cytometry, for example, for detecting leptomeningeal metastasis of solid tumors in liquor (Milojkovic Kerklaan *et al.*, 2016) or isolating epithelial cells from normal and tumor tissue (Bantikassegn *et al.*, 2015; Sinha and Lowell, 2016).

Fibroblasts represent a heterogeneous cell population and display an inconsistent expression of surface markers (Lynch and Watt, 2018). Here, isolation of fibroblasts was based on detection of CD90 and PDGFRα and binding of an anti-fibroblast antibody (Miltenyi, exact antigen is unknown). A cell was defined as 'fibroblastic' if positive for at least two of the three surface markers. CD90 has previously been used as a surface marker to isolate (cancer-associated) fibroblasts in gastrointestinal mucosa and ovarian cancer tissue (Gedye *et al.*, 2014; Kisselbach *et al.*, 2009) and also serves as a widely expressed mesenchymal surface marker (Jiang and Rinkevich, 2018). Likewise, PDGFRα is described as a membrane antigen of mesenchymal cells (Houlihan *et al.*, 2012) and supposed to participate in the recruitment of fibroblasts, pericytes, and endothelial cells during wound healing (Horikawa *et al.*, 2015). There is evidence for a successful enrichment of fibroblasts in flow cytometry using PDGFRα as a surface marker (Pallangyo *et al.*, 2015).

A total of 31 cell populations were successfully separated via flow cytometry and passed RNA-seq for further analysis. Table 2 illustrates all samples which underwent the complete algorithm.

We used gene ontology analysis, PCA, and RCA as well as population-specific expression of marker genes to demonstrate the successful separation of the three cell types.

3.3. Principal component analysis

To confirm an obvious separation of the cell populations, we used dimensionality reduction (PCA) to summarize the data into two dimensions and then visually identify obvious clusters. Cell types sorted by flow cytometry that have similar expression profiles were clustered together. Based on the PCA plot, there were three clean clusters corresponding to the different expected cell types and the first two principal components explain most of the variability (Fig. 3). The PCA supports a successful separation in three different cell populations.

3.4. Gene ontology analysis

Gene ontology (GO) is a widely used method to structure a large number of genes in certain categories (GO terms) describing biological processes, molecular functions, or cell components. Based on differential gene expression (Table S2) between the three cell populations, we created a list of the top upregulated genes for each cell type and matched them to specific GO terms of biological processes in which they were significantly overrepresented (FDR < 0.05). In the following, we list the top five GO terms for each sorted cell fraction, and for a complete overview, we refer to the Table S2.

Cells sorted for 'immune cell population' showed an enrichment in the categories immune system process, immune response, regulation of immune system process, regulation of immune response, and cell activation, whereas sorted cells of the 'fibroblast cell population' were enriched for extracellular matrix organization, anatomical structure morphogenesis, extracellular structure organization, multicellular organism development, and anatomical structure development. Those cells of the 'epithelial cell population' had highly expressed genes in GO terms tissue development, epithelium development, epidermis development, epithelial cell differentiation, and cornification.

Gene ontology analysis illustrated expected cell type-specific GO terms of biological processes for the three sorted cell populations, indicating a successful enrichment of the respective target cell types.

3.5. Marker gene analysis

To further confirm the separation of the three cell types, we considered marker genes of our target cell populations and evaluated their distribution within the differently expressed gene lists. Within the immune cell population, there was a significant upregulation of

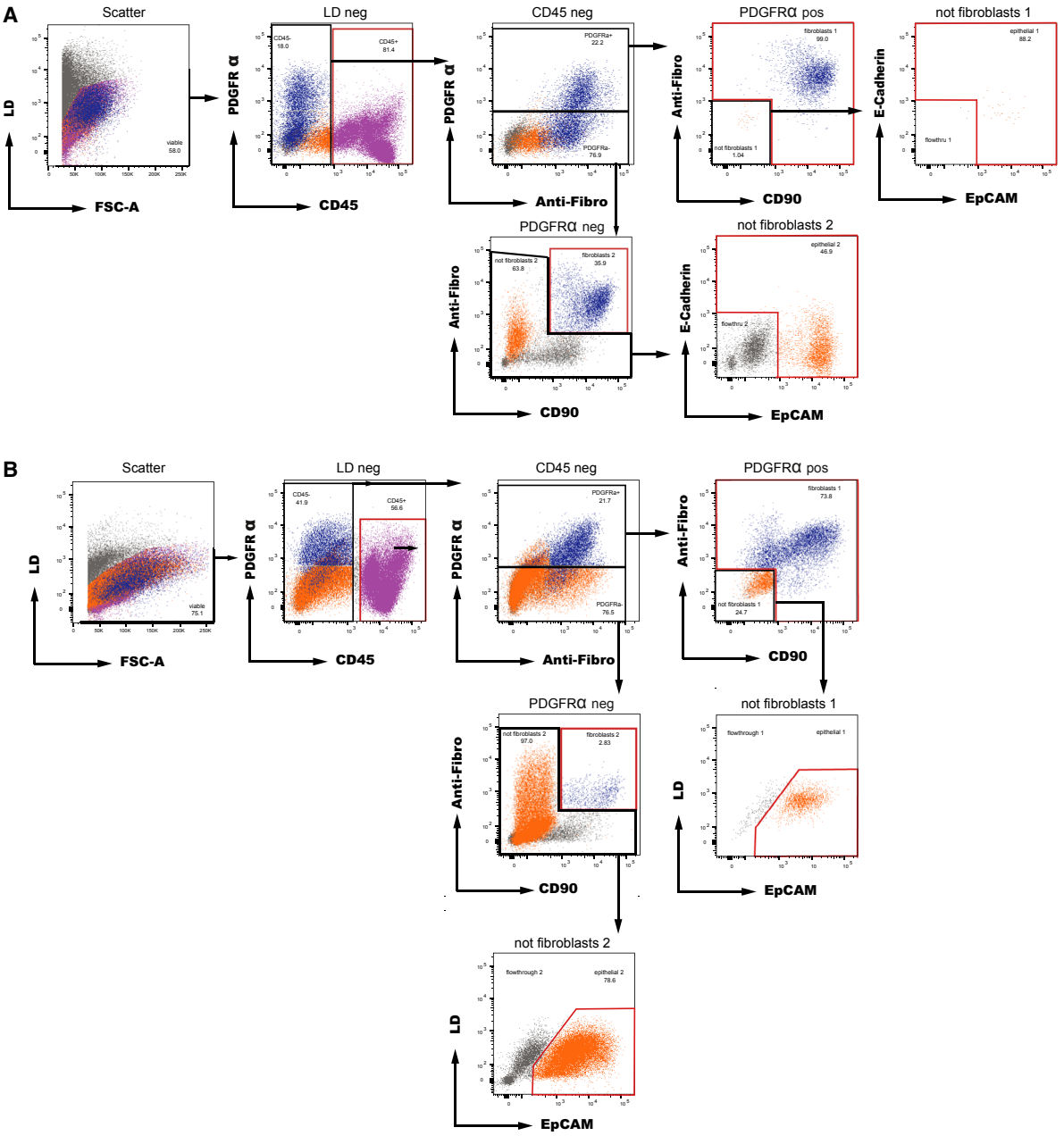

Fig. 2. Representative sample processing for separation of EAC cell populations from endoscopic biopsies by flow cytometry sorting with E-Cadherin co-staining (A) and without E-Cadherin co-staining (B). After initial viability gating, cells were separated into the (a) CD45+ immune cell population (pink); (b) fibroblast cell population (blue), defined as positive for at least two out of three of PDGFRα, anti-fibroblast, and CD90; and (c) epithelial cell population (dark orange) that were positive for EpCAM and/or E-Cadherin (A) or simply positive for EpCAM (B). The sorted cell subpopulations are highlighted in red.

surface proteins CD3 (gene name: *CD3D*), CD4 (*CD4*), CD8 (*CD8A* + *CD8B*), CD19 (*CD19*), and CD20 (*MS4A1*), all established markers for B/T lymphocytes. Furthermore, monocyte marker CD14 (*CD14*) was significantly upregulated as well as granulocyte marker CD11b (*ITGAM*).

In the fibroblast cell population, there was a significant increase in fibroblast activation protein (*FAP*), commonly described as a marker for (myo)fibroblasts (Shiga *et al.*, 2015). FAP is a surface peptidase with mostly unknown substrates which is involved in numerous physiological processes such as inhibition of

Table 2. Origin of all cell populations included for further RNA-seq.

		Successful RNA-seq of following cell populations after FACS sorting			
Patient No.	Sample origin	Tumor tissue	Sample labeling	Normal tissue	Sample labeling
1	Endoscopic biopsy	• Immune cells • Epithelia • Fibroblasts	• Tu1_immune • Tu1_epithel • Tu1_fibro	• Immune cells	• Mu1_immune
2	Endoscopic biopsy	• Immune cells • Epithelia • Fibroblasts	• Tu2_immune • Tu2_epithel • Tu2_fibro	–	–
3	Endoscopic biopsy	• Immune cells • Epithelia • Fibroblasts	• Tu3_immune • Tu3_epithel • Tu3_fibro	• Immune cells	• Mu3_immune
4	Endoscopic biopsy	• Immune cells • Epithelia • Fibroblasts	• Tu4_immune • Tu4_epithel • Tu4_fibro	• Immune cells • Epithelia	• Mu4_immune • Mu4_epithel
5	Endoscopic biopsy	• Immune cells • Fibroblasts	• Tu5_immune • Tu5_fibro	–	–
6	Surgical specimen	[a]	[a]	• Immune cells • Epithelia	• Mu6_immune • Mu6_epithel
7	Endoscopic biopsy	• Immune cells • Epithelia • Fibroblasts	• Tu7_immune • Tu7_epithel • Tu7_fibro	[a]	[a]
8	Surgical specimen	[a]	[a]	• Immune cells • Fibroblasts 1 • Fibroblasts 2	• Mu8_immune • Mu8_fibro1 • Mu8_fibro2
9	Surgical specimen	• Immune cells • Epithelia • Fibroblasts	• Tu9_immune • Tu9_epithel • Tu9_fibro	• Immune cells • Fibroblasts	• Mu9_immune • Mu9_fibro
Total		$N = 20$		$N = 11$	

[a]No samples taken.

fibrinolysis (Hamson *et al.*, 2014). In addition, we observed an upregulation of alpha-smooth muscle actin/alpha SMA (*ACTA2*) expression, characteristic for fibroblastic cells and which has been widely described in CAFs (Sharon *et al.*, 2013). We further observed in the fibroblast population a significant upregulation of expression of collagen genes (e.g., *COL1A1 and COL3A1*), responsible for the formation of the extracellular matrix (Yue, 2014).

E-Cadherin (*CDH1*) is a key epithelial marker, responsible for an epithelial barrier between neighboring cells via binding to adjacent cadherins and cytoskeleton (Serrano-Gomez *et al.*, 2016). *CDH1* was significantly upregulated in the sorted epithelial cells. Similarly, we found a significant increase in expression of epithelial membrane antigen (*EMA*, also known as *MUC1*) in this population. EMA is expressed in various epithelia and responsible for a physical barrier and anti-adhesive property of the tissue (Nath and Mukherjee, 2014).

In conclusion, cell type-specific genes showed a significant enrichment in their corresponding target cell populations, supporting the expected cell separation.

3.6. Reference component analysis

Reference component analysis is used to compare gene expression data sets to reference cell lineages. We compared each of the 31 sorted cell fractions by RCA with reference data (Fig. 4). RCA indicated consistent accordance to reference transcriptome data within equally sorted cells. There were three differential mapping (transcriptome correlation) patterns, each belonged to one of the three sorted cell types ('immune cell population', 'fibroblast cell population', 'epithelial cell population'), confirming the qualitative sufficiency of the separation process.

Cells sorted as 'immune cells' showed a high correlation with reference gene expression of inflammatory cells, for example, natural killer cells, T cells, B cells,

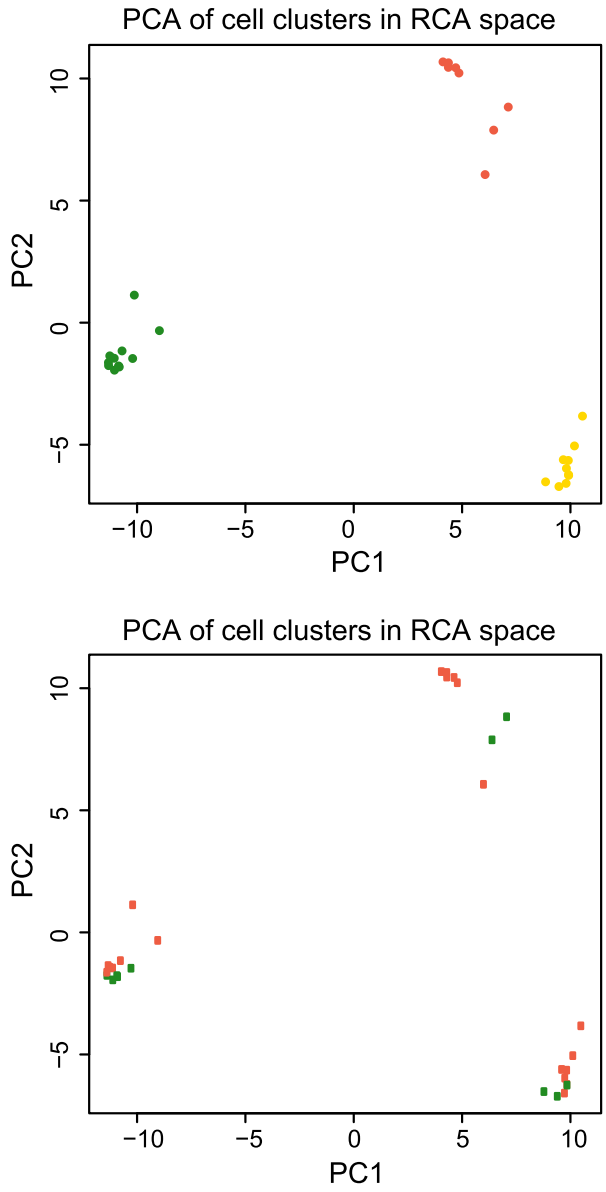

Fig. 3. Principal component analysis of RNA-seq of flow cytometry-sorted cell fractions. Thirty-one cell fractions were 3′RNA-sequenced and plotted by their first two principal components. Top: orange, epithelial cells; yellow, fibroblasts; green, leukocytes. Bottom: orange, tumor cells; green, normal cells.

monocytes, and macrophages. Cells of the 'epithelial cell population' matched to epithelial cell lines of bladder, lung, and colon and non-specific cells derived from tissue of pancreas and colon. Further correlations were found for trachea and intestinal tissue. Cells sorted as 'fibroblasts' revealed correlation with fibroblast cell lines, various mesenchymal stem cell lines, adipocytes, hematopoietic stem cells, endothelial cells, and smooth muscles cells (and organs with high proportion of smooth muscle cells, e.g., uterus). Cancer-associated fibroblasts (CAFs) have been reported to arise from various origins, for example, normal fibroblasts, adipocytes, bone marrow-derived cells (including mesenchymal stem cells), and endothelial cells (CD31+) (Wang *et al.*, 2016). Correlations with transcriptome data of these differential cell types might reflect the heterogeneous origin of the fibroblastic cells.

We explored how similar our cell type bulk sequencing was compared with scRNA-seq and co-analyzed esophagus epithelial scRNA-seq data of Owen *et al.* (2018) with epithelial cell types of our analyses. We observed largely congruent correlations with reference transcriptomes (Figs S1 and S2) indicating that the cell type bulk sequencing is pure enough for a representative snapshot of these cells. As expected, smaller cell populations with individual expression profiles can only be seen in scRNA-seq. Of note, the cell type-specific sequencing detected more genes per sample compared with scRNA-seq per cell (average 12 142 vs. 3260 to 4391 genes with ≥ 1 read).

3.7. Differently expressed genes between EAC and normal esophageal mucosa

Although the number of tumor–normal pairs does not allow general interpretations, we exploratively compared differentially expressed genes between EAC tissue and normal esophageal mucosa for each sorted cell population (Table S3). We first used GO analysis to categorize generic changes. Only the tumor/normal comparison of the fibroblast cell population showed significant enrichment of GO categories. Interestingly, blood vessel development, angiogenesis, and vasculature development were among the top 10 significantly enriched categories of biological processes (Table S4). These categories remained significant when restricting the analysis to upregulated genes, while downregulated genes were strongly enriched for the establishment of protein localization to endoplasmic reticulum, cotranslational protein targeting to membrane, and regulation of developmental process (Table S4). When focusing on individual genes with reported functions, the fibroblastic population showed strong upregulation of matrix metalloproteinase 11 (*MMP11*) in tumors (adjusted $P = 1.6 \times 10^{-15}$, Fisher's exact test). MMP11 serves as an endopeptidase-degrading extracellular matrix (Gómez-Macías *et al.*, 2018). *MMP11* overexpression in CAFs and cancer cells has previously been described to correlate with an aggressive cancer profile and promotion of metastasis (González de Vega *et al.*, 2019; Peruzzi *et al.*, 2009). Further, an upregulation of

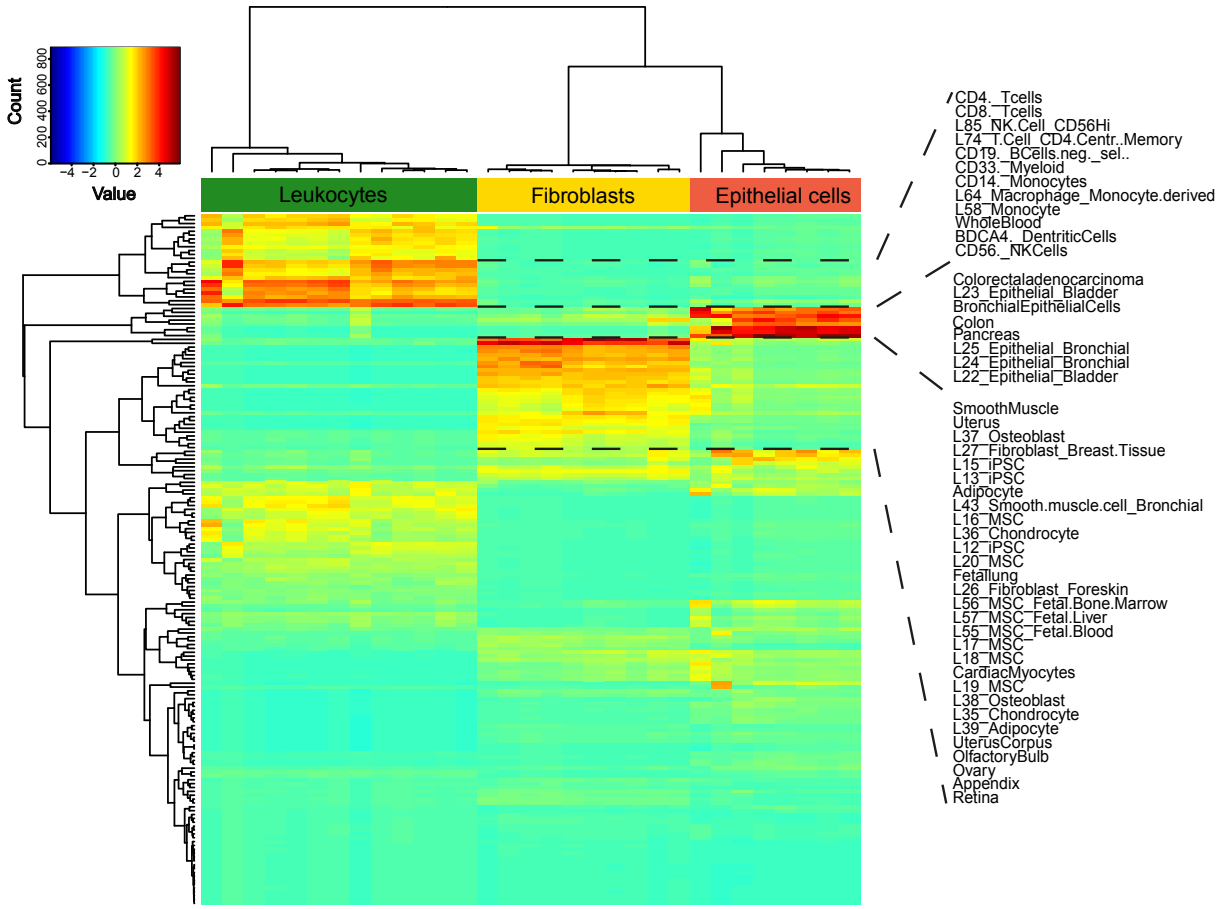

Fig. 4. Reference component analysis heat map of the cell populations' transcriptomic profiles correlated with expression profiles of reference tissues and cell lineages. RNA-seq data of 31 cell fractions plotted in columns with their color-coded Spearman's correlation values relative to reference expression datasets represented in rows. Cluster color code corresponds to colors in Fig. 3.

glycoprotein *CD38* was detected in the sorted immune cells from EAC tumors ($P = 8.6 \times 10^{-7}$, Fisher's exact test). CD38, usually expressed on plasma cells and other lymphoid and myeloid cell populations (Morandi *et al.*, 2018), has been revealed to mediate immunosuppression as a tumor escape mechanism, and there is evidence for an unfavorable CD38 influence on tumor progression in esophageal cancer (Chen *et al.*, 2018; Karakasheva *et al.*, 2015).

4. Discussion

In an effort to create a scalable approach for cell type-specific comparison of tumors, we have developed a protocol to dissociate fresh biopsies of EACs and corresponding esophageal mucosa, and sort leukocytes (CD45+), epithelial cells (EpCAM+), and fibroblasts (positive for at least two out of PDGFRα, CD90, anti-fibroblast), followed by 3′RNA sequencing. This workflow allows us to investigate the transcriptomic changes in the three cell populations in EAC when compared to normal esophageal mucosa. Importantly, this approach is significantly more economic and scalable for investigating transcriptomes of different cell types compared with single-cell RNA sequencing (scRNA-seq) and can therefore serve as a cost-effective alternative to broaden understanding of tumor biology in EAC. In our local setting, the costs for the analysis of a tumor/normal pair with three cell types each by scRNA-seq (hashing for tumor and normal) would be 3.2 times higher compared with cell type-specific RNA-seq.

In order to prove the successful workflow of this approach, we demonstrate a clear separation of our three target cell populations (fibroblasts, immune cells, and epithelial cells) and performed 3′RNA sequencing of these sorted cell types. We used different strategies to verify the identity of the cells. GO analysis elucidated functionally compatible and expected biological

processes for the respective cell populations. We provide further evidence for a successful separation by comparing transcriptomic profiles with reference expression data using RCA and obtained three different expression correlation patterns, each specific for the respective target population: Cells sorted for epithelial cell compartment showed high correlation with epithelial cell lineages, the immune cell compartment with reference data of differential lymphocytes, monocytes, and granulocytes and fibroblasts with fibroblasts, various mesenchymal cell lines, and cell lineages that can be considered plausible to serve as origins of CAFs. Literature-based marker gene analysis within differentially expressed genes further supported a qualitative separation into the three cell compartments.

We focused on optimizing the protocol for small amounts of tissue, allowing to obtain the samples endoscopically at primary staging. Up-scaling of our protocol will enable us to compare biopsies of treatment-naïve EAC tissue with normal esophageal mucosa of patient cohorts at moderate cost. Such systematic cell type-specific analysis of treatment-naïve, endoscopically obtained samples and correlation with clinical features will allow to find new cell type-specific pathway alterations, responsible, for example, for treatment response or relapse.

Although we show a potential application of our protocol for biopsies and surgical specimens in normal esophageal mucosa and EAC tissue, the protocol can be further improved. Especially, freezing of dissociated single-cell suspensions, necessary due to processing logistics, was responsible for a substantial loss of living cells. A continuous workflow without freezing will improve the cell viability and quantity of RNA for sequencing. Since the different samples have varying proportions of the three cell populations, we were not able to obtain sufficient cells for all sorted cell populations. This may explain the lack of significantly enriched GO categories for the leukocyte and epithelial compartments. An increase in the number of viable cells by a continuous workflow will enhance the chance to robustly obtain sufficient amounts of RNA for subsequent 3′RNA-seq.

In order to demonstrate the potential utility of our approach, we finally compared differently expressed genes between normal esophageal mucosa and EAC tissue and highlighted cell type-specific alterations. For instance, *CD38* upregulation in immune cells of EAC could establish a possible target of immunotherapy, as suggested in combination with anti-PD-L1 therapy (Chen *et al.*, 2018; Mittal *et al.*, 2018). For *MMP11*, which is usually expressed both in epithelial cells and fibroblasts, we found an upregulation restricted to the fibroblast cell population as described earlier (Pedersen *et al.*, 2009). Although sample numbers used here are limited, it is interesting to note that fibroblasts in the tumor showed upregulation of angiogenesis genes, when compared to normal mucosa. Angiogenesis-promoting properties of CAFs have been described earlier (reviewed in Wang *et al.*, 2019) and are an area of intense investigation in the search for new angles of intervention. Future implementation of the protocol in a large cohort of patients can therefore help to identify targets for novel therapy concepts and complement our understanding of tumor biology in EAC.

In conclusion, we present a new approach for a cost-effective and scalable procedure to determine cell type-specific transcriptome alterations in treatment-naïve EAC tissue with the potential to obtain treatment-relevant findings in large cohorts of patients with EAC.

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Author contributions

MK, PSP, AQ, HS, S-HC, and AMH designed the study. MK, PSP, KF-D, MT, IG-M, CW, SB, JW, MF, and JA carried out the experiments. PSP, HS, WS, CJB, HA, and S-HC collected and selected the patients' samples. HL, AQ, and RB performed the histopathological examinations. OVC performed bioinformatic analyses. MK, PSP, OVC, CW, AQ, and AMH interpreted the data. MK, PSP, OVC, and AMH wrote the manuscript with contributions of CW and KF-D and approval from all authors.

Conflict of interest

MK is supported by the Koeln Fortune Program/Faculty of Medicine, University of Cologne, grant number 410/2018. PSP is fellow of the Else Kröner Forschungskolleg Cologne 'Clonal Evolution in Cancer' (2016-Kolleg-19). All other authors declare no conflict of interest.

Data accessibility

The RNA-seq data have been deposited at the European Genome-Phenome Archive and can be accessed via the accession number EGAS00001004053.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Joint normal esophageal mucosa RCA of scRNA-seq and cell type RNA-seq.

Fig. S2. Normal esophageal mucosa RCA of pseudo-bulk scRNA-seq.

Table S1. Complete lists of DEGs in a pairwise manner.

Table S2. List of the top five Gene Ontology terms for each sorted cell fraction.

Table S3. Differentially expressed genes between EAC tissue and normal esophageal mucosa for each sorted cell population (Gene Ontology Analysis).

Table S4. Top ten significantly enriched categories of biological processes between EAC tissue and normal esophageal mucosa for each sorted cell population (Gene Ontology Analysis).

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ORIGINAL RESEARCH

The expression of the immune checkpoint regulator VISTA correlates with improved overall survival in pT1/2 tumor stages in esophageal adenocarcinoma

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ABSTRACT

Immune checkpoint modulation in cancer has been demonstrated as a high-value therapeutic strategy in many tumor entities. VISTA is an immune checkpoint receptor regulating T-cell function. To the best of our knowledge, nothing is known about the expression and prognostic impact of VISTA on tumor infiltrating lymphocytes (TILs) in the tumor microenvironment of esophageal adenocarcinoma (EAC). We analyzed in total 393 EACs within a test-cohort (n = 165) and a validation-cohort (n = 228) using a monoclonal antibody (clone D1L2G). These data were statistically correlated with clinical as well as molecular data. 22.2% of the tumor cohort presented with a VISTA expression on TILs. These patients demonstrated an improved median overall survival compared to patients without VISTA expression (202.2 months vs. 21.6 months; $p < 0.0001$). The favorable outcome of VISTA positive tumors is significant in the entire cohort but mainly driven by the general better prognosis of T1/T2 tumors. However, in the pT1/2 group, VISTA positive tumors show a tremendous survival benefit compared to VISTA negative tumors revealing real long-term survivors in this particular subgroup. The survival difference is independent of the T-stage. This unique characteristic could influence neoadjuvant therapy concepts for EAC, since a profit of therapy could be reduced in the already favorable subgroup of VISTA positive tumors. VISTA emerges as a prognostic biomarker for long-term survival especially in the group of early TNM-stages. Future studies have to show the relevance of VISTA positive TILs within a tumor concerning response to specific immune checkpoint inhibition.

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Introduction

Esophageal cancer is the eighth most common malignant tumor worldwide and the number of incidences of esophageal adenocarcinoma (EAC) is increasing especially in the Western world (see http://www.wcrf.org). The majority of adenocarcinomas arise from Barrett metaplasia due to chronic reflux disease, followed subsequently by an accumulation of different mutations causing genetic instability (Barrett multistep carcinogenesis).[1,2] Frequently patients present with a locally advanced tumor stage. Despite improvements in perioperative treatments, the overall survival rates of patients with esophageal adenocarcinoma remains poor.

To evade immune-control, virtually all fully developed tumors are associated with an immunosuppressive tumor microenvironment with elevated levels of tumor associated macrophages (TAMs) and regulatory T-cells (Tregs). The immune-escape of tumor cells itself is facilitated e.g. by a loss of specific antigens and tumor associated immunosuppressive cells preventing T-cell activation. Concurrent, infiltrating effector T-cells develop tolerance against the tumor cells. This is facilitated by co-inhibitory receptors- so called immune checkpoints that are able to modulate the effector T cell function. This has led to numerous studies identifying therapeutically targets for immune checkpoint modulation.

V-domain Ig suppressor of T cell activation (VISTA) is an immune checkpoint receptor expressed on tumor infiltrating T-lymphocytes (TILs) and myeloid cells, leading to suppression of T-cell activation, proliferation and cytokine production.[3] The extracellular domain is similar to that of PD-L1, although both proteins interfere with different subsets of T-lymphocytes.

In the here presented study, we analyzed the hypothesis, that elevated numbers of VISTA-positive TILs in esophageal adenocarcinomas are associated with differences in prognosis. Therefore, the number of VISTA positive TILs was assessed on TMAs of two independent cohorts of esophageal adenocarcinoma using immunohistochemistry.

Results

Clinico-pathological and patients characteristics

Patient characteristics are given in Tables 1 and 2. 393 patients with esophageal adenocarcinomas (EAC) that underwent surgical tumor resection were immunohistochemically interpretable

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Table 1. Patient's characteristics and VISTA expression results on test-cohort (n = 165). Total patient´s numbers do not add to n = 165 due to missing analysable tumor spots on the multi-spot TMA.

			VISTA expression surface margin					VISTA expression infiltration margin				
			low		high			low		high		
sex	No	%	No	%	No	%	*p* value	No	%	No	%	*p* value
female	16	9.7%	13	81.3%	3	18.8%		11	73.3%	4	26.7%	
male	149	90.3%	110	77.5%	32	22.5%	0.508	104	72.2%	39	27.3%	0.614
age group												
<65 years	72	43.9%	55	78.6%	15	21.4%		51	73.9%	18	26.1%	
>65 years	92	56.1%	67	77.0%	20	23.0%	0.485	63	71.6%	25	28.4%	0.444
tumor stage												
pT1	49	30.1%	28	60.9%	18	39.1%		29	63.0%	17	37.0%	
pT2	29	17.8%	18	62.1%	11	37.9%		20	69.0%	9	31.0%	
pT3	84	51.5%	76	93.8%	5	6.2%		65	80.2%	16	19.8%	
pT4	1	0.6%	0	0.0%	1	100%	<0.0001	0	0.0%	1	100%	0.062
lymph node metastasis												
pN0	63	38.7%	34	56.7%	26	43.4%		35	59.3%	24	40.7%	
pN1	72	44.2%	65	92.9%	6	7.1%		57	81.4%	13	18.6%	
pN2	13	8%	10	83.3%	2	16.7%		11	84.4%	2	15.4%	
pN3	15	9.2%	13	86.7%	2	13.3%	<0.0001	11	73.3%	4	26.4%	0.030
UICC stage												
I	41	26.1%	21	51.2%	20	48.8%		23	57.6%	17	42.5%	
II	21	13.4%	15	71.4%	6	28.6%		14	66.7%	7	33.3%	
III	75	47.8%	69	92.0%	6	8.0%		60	78.9%	16	21.1%	
IV	20	12.7%	17	85.9%	3	15.0%	<0.0001	17	85.0%	3	15.0%	0.045

Table 2. Patient's characteristics, VISTA expression results, HER2- and p53-status on validation cohort (n = 393).

				VISTA Expression				
		No	%	low		high		*p* value
sex	female	40	10,2%	27	67.5%	13	32.5%	
	male	353	89,8%	252	71.4%	101	28.6%	0.364
age group	<65 yrs	203	51,7%	141	69.3%	62	30.7%	
	>65 yrs	190	48,3%	140	73.4%	50	26.6%	0.224
tumor stage	pT1	43	10,9%	22	51.2%	21	48.8%	
	pT 2	35	8,9%	27	77.1%	8	22.9%	
	pT 3	305	77,6%	223	73.3%	82	26.7%	
	pT 4	10	2,5%	6	60.0%	4	40.0%	0.017
lymph node metastasis	pN0	148	37,7%	94	63.5%	54	36.5%	
	pN 1	155	39,4%	111	71.4%	44	28.6%	
	pN 2	46	11,7%	35	76.1%	11	23.9%	
	pN 3	44	11,2%	39	88.6%	5	11.4%	0.010
UICC stage	I	70	17,8%	40	57.1%	30	42.9%	
	II	70	17,8%	46	66.1%	24	33.9%	
	III	160	40,7%	115	71.9%	45	28.1%	
	IV	92	23,4%	77	83.8%	15	16.2%	0.008
TP53	wildtype	150	41.0%	108	72.2%	42	28.0%	
	mutation	216	59.0%	153	70.8%	63	29.2%	0.451
HER2	wildtype	307	87.7%	215	70.0%	92	30.0%	
	amplification	43	12.3%	33	76.7%	10	23.3%	0.236

on the single-spot and 165 patients on the multi-spot tissue micro array (TMA). Reasons for non-informative cases (35 spots; 8.2%) included a lack of tissue samples or an absence of unequivocal cancer tissue in the TMA spot.

VISTA expression (in test and validation cohort)

VISTA immunostaining was localized in the cytoplasm/membrane of tumor infiltrating lymphocytes (Figure 1(a+b)). In total, with the applied scoring system, 22.2% (n = 35) of patients on the multi-spot TMA were considered VISTA positive. In 104 (63.0%) cases we found a heterogeneous expression of VISTA within the 4 spots of the two localizations (surface and invasive margin) (Figure 1(c–f); Figure 2). On the other hand, the overall expression pattern of the surface compared with the invasive tumor margin showed only a low heterogeneity, thus 18 cases (11.6%) were positive for VISTA on the surface and not on the invasive margin. In cross-table analysis, high amounts of VISTA-positive TILs were correlated with early (pT1/2) tumor stages ($p < 0.0001$), nodal negative patients ($p < 0.001$) and early UICC stages (UICC stage I/II) ($p = 0.004$) (Table 1).

In 1.2% of the analyzed cases we found an expression of VISTA on carcinoma cells.

In the validation cohort (on the single-spot TMA), 114 patients were considered VISTA positive (29.0%). We found comparable results to the multi-spot TMA, especially the strong correlation of VISTA to early tumor stages (pT1/2) ($p = 0.017$), nodal negative patients ($p = 0.010$) and UICC stages (UICC stage I and II) ($p = 0.008$) (Table 2).

VISTA expression reveals long-term survivors in EAC

Prognostic significance with respect to overall survival was seen for VISTA expression as determined by Kaplan-Meier survival

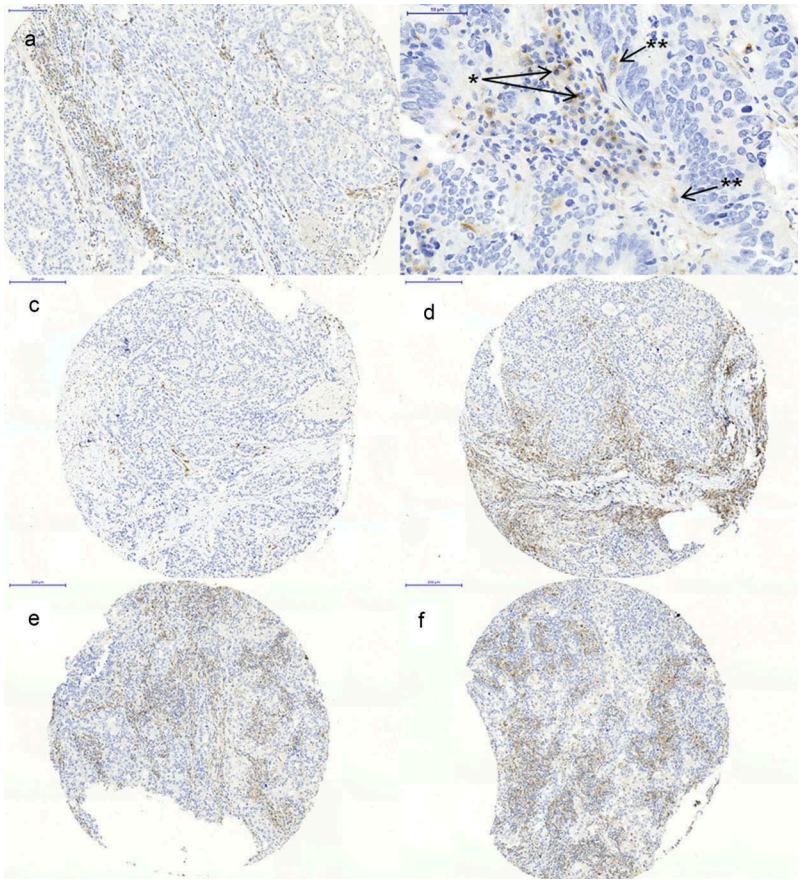

Figure 1. Immunohistochemistry of VISTA. (a) high expression of VISTA on TILs; (b) VISTA expression on lymphocytes (*) and macrophages (**); (c+d) tumor spots of the same tumor showing heterogeneous low and high VISTA expression; (e+f) tumor spots of the same tumor showing homogeneous VISTA expression.

analysis. For both, expression on the surface and infiltration margin, VISTA expression was correlated with favorable outcomes in patients with EAC (Figure 3(a,b)). Calculated median overall survival in patients with VISTA expression on the surface margin was 202.2 months (95% confidence interval (CI) 32.6–371.8 months) compared to a median OS of 21.6 months in VISTA negative patients (95%CI 13.3–29.9 months) ($p < 0.001$). Similar results were found on the invasive margin. The median overall survival in VISTA positive patients was 84.6 months (95% CI 27.2–141.9 months) compared to 22.1 months (95%CI 15.1–-29.0 months) in VISTA negative patients ($p = 0.024$). In subgroup analysis, tumors with VISTA-positive TILs demonstrate a superior overall survival in early tumor stages (pT1/2) compared to patients without VISTA expression on TILs ($p < 0.003$) (Figure 3(c)). The survival benefit is not seen in higher tumor stages (Figure 3(d)). Due to the fact of highly homogenous distribution of VISTA within the tumor (irrespective of tumor surface or tumor infiltration margin), we do not consider the surface or infiltration margin in the single spot TMA of the validation cohort anymore. We were able to confirm the prognostic power of VISTA on the validation cohort considering 228 additional patients. For the entire validation cohort, VISTA expression was associated with a prolonged overall survival with a median overall survival of 41.9 months (95% CI 18.0–65.9 months) vs. 25.7 months in VISTA negative patients (95% CI 19.1–32.3 months) ($p = 0.046$) (Figure 3(e)). In subgroup analysis adjusted for tumor stages, similar results as already described for the multi-spot TMA were revealed with a survival benefit especially in the pT1/2 group, where VISTA positive tumors represent real long-term survivors in this particular subgroup (Figure 3(f)). The survival difference is independent of the T-stage (Table 3).

Correlation of VISTA expression in subgroups of tils

To visualize which subtypes of TILs express VISTA we performed double stain immunofluorescence with VISTA and CD4, CD8 and CD68. A semiquantitative analysis was performed on 40 VISTA positive cases correlating VISTA and CD4, CD8 and CD68. VISTA showed a predominant co-expression with CD68, and in the subgroup of TILs a co-expression with CD4 in most cases (Figure 4). No reliable co-expression with CD8 was detectable.

Correlation of VISTA expression with other biomarkers of the tumor-microenvironment

We correlated the VISTA expression on TILs with the expression pattern of PD-L1 (on tumor cells), LAG3, CD3 and CD8 on TILs (these data currently under review). In the cohort of EAC LAG3 is associated with a better outcome, whereas PD-L1 showed no prognostic impact. We were not able to find any statistically relevant correlation of the aforementioned markers in the present patient's cohort. No survival differences were

Figure 2. Heat-map of VISTA distribution within the multi spot TMA displaying heterogeneous expression. Each line represents one particular patients, each column represents one spot on the multi-spot TMA. Blue = negative VISTA expression (<1% of TILs), light red = low VISTA expression (1–4% of TILs), dark red = high VISTA expression (>4% of TILs).

observed for patients with low or high CD3 expression with respect to VISTA expression (Supplementary Figure 1).

VISTA expression and TP53 and HER2 status

TP53 mutation and HER2 amplification/expression status was available for the entire patient's cohort (data not shown). Within the VISTA positive group 63 patients showed a TP53 mutation (60.0%) and 42 patients (40.0%) were TP53 wild-type tumors ($p = 0.451$). Similar results were found for HER2 amplification. In tumors with high VISTA expression 10 patients showed HER2 amplification, but a correlation via cross-table analysis did not reveal a significant association between VISTA and HER2 amplification ($p = 0.236$).

Discussion

In a large set of 393 patients with EAC we report the expression of VISTA positive tumor associated lymphocytes (TILs) evaluating the level of heterogeneity and distribution within the tumor. In the patient cohorts, we are able to show a significantly favorable outcome for VISTA positive tumors in pT1/T2 stages and find generally a lower level of VISTA expression in pT3/T4 tumor samples. Furthermore, we do not find any correlation of VISTA with important molecular alterations like TP53 mutational status and HER2 amplification status, as well as with further important biomarkers of the tumor microenvironment like the number of T-cells (CD3) and previously examined LAG3 expression on TILs (Data are currently under revision).

We created a multi-spot TMA considering two different tumor localizations (surface, infiltration margin) as a test cohort,

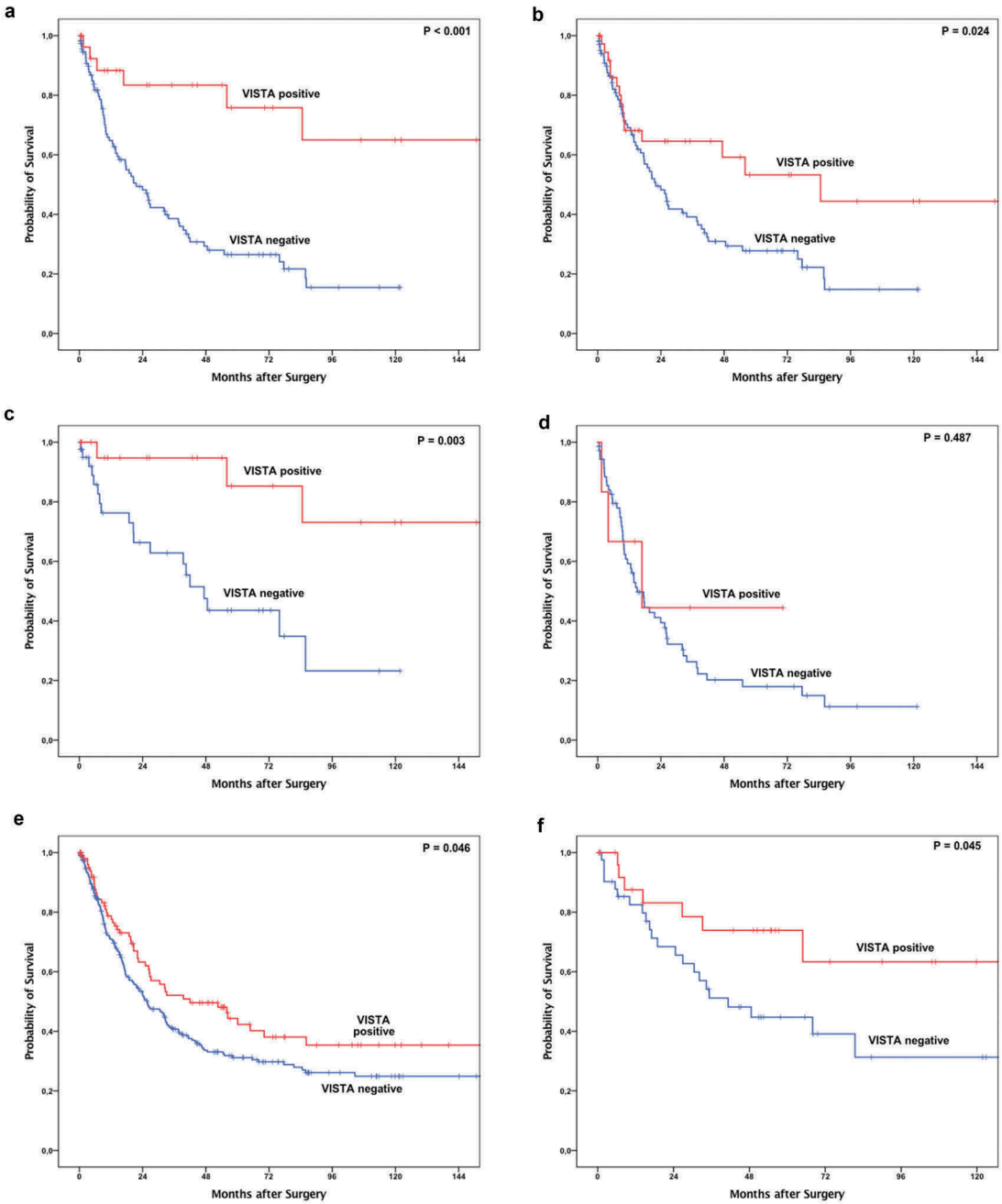

Figure 3. Kaplan-Meier survival analysis of VISTA on the multi spot TMA (n = 165 patients). In both, surface (a) and infiltration zone (b) of the tumor, VISTA expression on tumor infiltrating T-cells (TILs) is correlated with superior overall survival compared to VISTA negative TILs. In subgroup analysis, the survival benefit of VISTA expression reveals in early invasive tumor stages (pT1/2 (c)) and is not detectable in advanced tumor stages (pT3/4 (d)). A difference in overall survival is also detectable in the validation cohort (e) which is predominantly driven by the survival difference in early tumor stages (pT1/2 (f)).

Table 3. Multivariate cox-regression model for early invasive tumor stages (pT1 and pT2). HR = hazard ratio.

		95% confidence interval		
	HR	lower	upper	*p* value
sex (male vs. female)	1.421	0.377	5.357	0.603
age group (<65 vs. >65)	1.569	0.576	4.271	0.378
tumor stage (pT1 vs. pT2)	1.426	0.536	3.794	0.477
lymph node metastasis (pN0 vs. pN+)	2.751	0.923	8.2	0.069
VISTA expression	0.207	0.055	0.783	0.02

where we were able to prove a low heterogeneity of VISTA expression within the tumor. There is a consistent pattern between the tumor surface and the infiltration margin indicating that EAC samples taken by endoscopic tumor biopsy can represent overall tumor VISTA expression. Furthermore, the absence of significant heterogeneity was one reason to create a single-spot TMA with 228 additional patients as a validation cohort.

To the best of our knowledge we are the first describing the expression pattern of VISTA and its prognostic impact in EAC. Previously, Böger et al.[4] examined the role of VISTA in gastric cancer. Interestingly they found comparable results considering the cumulative distribution of VISTA in T1/T2 stages in opposite to T3/T4 – they describe a significant decrease of VISTA positive TILs between the pT2 to the pT3 stage.

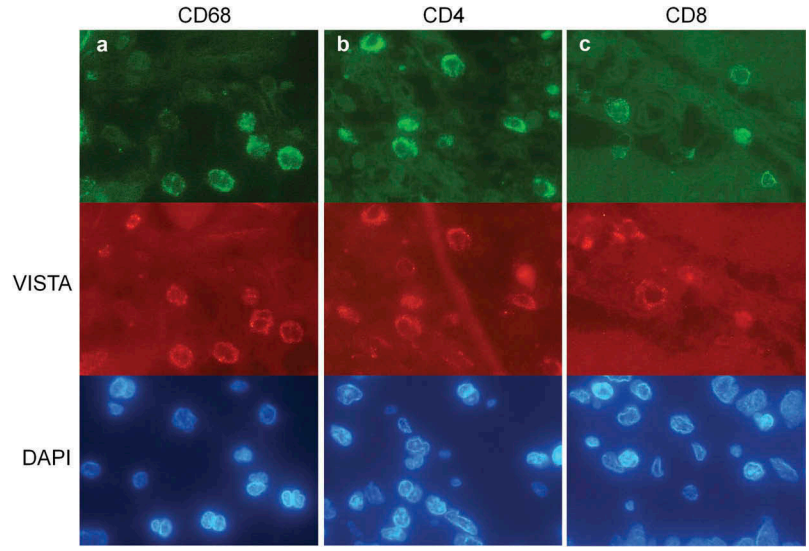

Figure 4. Double staining immunofluorescence of VISTA (red signals) and CD68, CD4 and CD8 (green signals) with counterstaining of the nuclei with DAPI (blue signals). The photos (a-c) represent one double staining each with different fluorescence filters. (a) VISTA and CD68 show a coexpression representing myeloid origin; (b) VISTA and CD4 show a strong coexpression within the VISTA positive TILs; (c) No correlation of VISTA and CD8 was seen.

In the analyzed cohort, VISTA expression significantly represents a positive prognostic marker in the subgroup of pT1/T2 stage tumors. In addition, the favorable outcome of VISTA positive tumors is even significant in the entire cohort but mainly driven by the general better prognosis of pT1/T2 tumors (VISTA – positive TILs in higher levels in T1/T2 than in T3/T4 stages). However, in the pT1/2 group, VISTA positive tumors show a tremendous survival benefit compared to VISTA negative tumors revealing real long-term survivors in this particular subgroup. The survival difference is independent of the T-stage. This unique characteristic could influence neoadjuvant therapy concepts for EAC, since a profit of therapy could be reduced in the already favorable subgroup of VISTA positive tumors. Further research in this context might contribute to build cohorts of profiting versus non-profiting groups of patients.

Nevertheless, the significantly lower expression of VISTA in pT3/T4 tumor stages remains cryptic. On the one hand, it is conceivable that changes in tumor biology are responsible for a loss/reduced amounts of VISTA-positive TILs in locally advanced tumors; on the other hand VISTA itself could even influence invasive tumor growth.

The exact physiological mechanism of action for VISTA is still obscure. It is assumed to suppress T-cell activity and serves as an immune checkpoint.[3] It might therefore play a role in the immune evasion of tumors. Some studies could provide evidence for this assumption. In oral squamous cell cancer, high VISTA expression in combination with low CD8 expression is associated with poor prognosis and lymph node metastases.[5] In mouse models, VISTA blockade impaired tumor growth by attenuating the tumor microenvironment.[3,6] In the here presented study VISTA expression was a significant and independently positive prognostic marker in the subgroup of pT1/T2 stage tumors. In a previous study of gastric cancer, VISTA expression was predominantly seen in pT1/T2-stages, although it did not correlate with prognosis.

While VISTA is displayed by antigen-presenting cells (APCs) and T-cells, Böger et al.[4] showed an expression on gastric carcinoma cells in 8.8%. In the cohort of esophageal adenocarcinoma, VISTA is only rarely present on carcinoma cells (1.2%).

The immune regulating functions of VISTA suggest that an elevated expression is attended by a highly inflamed tumor microenvironment. In opposite to this and previous results for PD-L1 in EAC, we were able to show an independent expression of VISTA concerning the number of T-cells (CD3).[7] We found a predominant co-expression of VISTA and CD68, underlining the known expression on myeloid cells.[8] On T-cells we were able to find a strong and convincing co-expression of VISTA and CD4-positive T-cells. We did not see a persuading co-expression with CD8-positive T-cells. This is in keeping with known data showing VISTA on CD8-positive T-cells to be expressed in a lower frequency and intensity, so we were probably not able to measure a discrete expression reliably by immunofluorescence.[9,10] The functional impact of VISTA-positive CD4-positive T-cells is not clear yet, but there is evidence for VISTA regulating T-cell function both as a ligand and receptor.[8,10,11] Anyway, we did not find any correlation with other checkpoint-markers (PD-L1, LAG3) in this cohort indicating that VISTA might function in a more independently manner. The exploration of alternative mechanisms of action, besides regulatory effects on inflammatory microenvironment, might lead to a better understanding of immunomodulation via VISTA in EAC.

Our study has a few limitations. The study is retrospective and a selection bias cannot be excluded. For example, we were not able to test patients who received neoadjuvant treatment and showed a complete tumor response. Functional data on the exact effect of VISTA-expression in EAC could unfortunately not be compiled on formalin fixed paraffin embedded (FFPE) tumor specimen. Our results need to be confirmed by further studies.

Conclusion

Our study reveals the prognostic significance of VISTA expression in esophageal adenocarcinoma. We find VISTA-

positive TILs in a significant number of EAC in this cohort, which correlates with improved overall survival within pT1/T2 stages. Thus, this subgroup might be a promising approach to improve personal targeted treatment decisions and lead to new perspectives on neoadjuvant therapy concepts in EAC.

Methods

Patients and tumor samples

In this retrospective study we analyzed 393 patients with esophageal adenocarcinomas that underwent primary surgical resection or resection after neoadjuvant therapy between 1999 to 2016 at the Department of General, Visceral and Cancer Surgery, University of Cologne, Germany. The recently published criteria for reporting recommendations for tumor marker prognostic studies (REMARK criteria) were followed in this study.[12,13]

According to the suggestions of the international immuno-oncology working group for assessing tumor infiltrating lymphocytes (TILs) in solid tumors we created a multi spot tissue micro array (TMA) with up to 12 tumor spots as a test-cohort considering formalin fixed and paraffin embedded (FFPE) material of 165 patients with EAC.[14] 148 patients (89%) of the test-cohort did not receive any neoadjuvant treatment. We considered equally the tumor surface/centre and the tumor infiltration margin where possible. Additionally we created a single spot TMA considering 228 additional patients as a validation-cohort (393 patients in total). The construction of the TMAs was performed as previously described.[15,16] In brief, tissue cylinders with a diameter of 1.2 mm each were punched from selected FFPE tumor tissue blocks using a self-constructed semi-automated precision instrument and embedded in empty recipient paraffin blocks. For the multi-spot TMA, up to 8 tumor spots were punched out of the tumor, four spots each from the endoluminal and the invasion front. These data were statistically correlated with molecular data like *TP53* mutational and the HER2 amplification status. Four µm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ) for immunohistochemistry. Standard surgical procedure was laparotomic or laparoscopic gastrolysis and right transthoracic en bloc esophagectomy with intrathoracic esophagogastrostomy including two-field lymphadenectomy of mediastinal and abdominal lymph nodes or transhiatal extended distal esophagectomy with transabdominal intrathoracic or cervical anastomosis as described previously.[17] Patients with advanced esophageal cancer (cT3, cNx, M0) received preoperative chemoradiation (5-FU, cisplatin, 40Gy) or chemotherapy. Follow-up data were available for all patients. Depending on the effect of neoadjuvant chemo- or radiochemotherapy, there is a preponderance of minor responders, defined as histopathological residual tumor of ≥10%.[18] All procedures followed the national and institutional ethical standards and were in accordance with the relevant version of the Helsinki Declaration. Informed and ethical approved consent (13-091) was obtained from all included patients.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on TMA slides. For VISTA the rabbit IgG monoclonal antibody (D1L2G; dilution 1:100; Cell Signalling Technology, Netherlands) was used. All immunohistochemical stainings were performed using the Leica BOND-MAX stainer (Leica Biosystems, Germany) according to the protocol of the manufacturers. The evaluation of immunohistochemical expression was separately assessed independently by two experienced pathologists (AQ and PL), blinded to clinical data. Discrepant results, which occurred in less than 10% of samples, were resolved by consensus review of the particular tumor spots.

Strategy of evaluation

VISTA is expressed on myeloid cells and lymphocytes. Only the expression on lymphocytes was evaluated. VISTA: <1% of lymphocytes was defined as negative, 1–4% of lymphocytes was assessed as "VISTA low", >4% of lymphocytes was counted as "VISTA high".

Concerning the multi-spot TMA four spots of tumor surface and invasive margin each were examined. We built the average of the scores and matched the four samples to one category based on limit values: 0–0.49 = negative, 0.5–1.49 = low, 1.5–2 = high.

(e.g.: VISTA expression in spot 1: 2, spot 2: 1, spot 3: 0, spot 4: 2, average of the spots: 1.25 → category "low").

Discrepant results were resolved by consensus review.

For the statistical analysis, high VISTA expression was assessed as positive and negative or low expression as negative.

Double staining immunofluorescence

Immunofluorescence was performed on TMA slides. For the immunofluorescence double staining, paraffin sections were deparaffinised and antigens were retrieved with citrat. Slides were incubated with the primary antibody (CD4, Thermo Scientific MS-1528 1:100; CD8, Dako M7103 1:100; CD68 Dako M0876 1:200; VISTA, Cell signalling 649535 1:100) followed by incubation with the appropriate secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) and counterstaining of the nuclei with DAPI (Sigma-Aldrich). Tumor tissue was scanned for VISTA positive cells (red signals) using a 63x objective (DM5500 fluorescent microscope; Leica). VISTA positive cells were counted and a co-expression with CD4, CD8 or CD68 (green signals) was assessed.

TP53 status

The immunohistochemical TP53 status was correlated with the *TP53* mutational status using parallel sequencing. A detailed description was recently published (Quaas A et al., Genomic characterization of TP53 wild type esophageal carcinoma, Translational Oncology, in press). In brief, the tumor DNA was extracted, amplified with a customized GeneRead DNAseq Targeted Panel V2 (Qiagen), libraries were constructed and quantified, and exons 5–8 of the *TP53 gene* were sequenced on the MiSeq (Illumina). A 5% cut-off for variant calls was used and results were only interpreted if the coverage was >200x.

Statistical analysis

Clinical data were collected prospectively according to a standardized protocol. SPSS Statistics for Mac (Version 21, SPSS) was used for statistical analysis. Interdependence between staining and clinical data was calculated using the chi-squared and Fisher's exact tests, and displayed by cross-tables. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were performed for prognostic factors of overall survival using the Cox regression model. All tests were two-sided. P values <0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE – CANCER RESEARCH

Lymphocyte activation gene-3 (LAG3) mRNA and protein expression on tumour infiltrating lymphocytes (TILs) in oesophageal adenocarcinoma

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Abstract

Purpose Lymphocyte activation gene-3 (LAG3) is an immunosuppressive checkpoint molecule expressed on T cells. The frequency and distribution of LAG3 expression in oesophageal adenocarcinoma (EAC) is unknown. Aim of the study was the evaluation and distribution of LAG3 on tumour infiltrating lymphocytes (TILs) and correlation with clinico-pathological and molecular data.

Methods We analysed tumor tissue samples using immunohistochemistry, multi-colour immunofluorescence and mRNA in-situ technology. The analyses were performed on a multi-spot tissue microarray (TMA) with 165 samples, followed by an evaluation on a single-spot TMA with 477 samples. These results were correlated with clinical and molecular tumour data.

Results LAG3 expression on TILs was detectable in 10.5% on the multi-spot TMA and 11.4% on the single-spot TMA. There was a strong correlation between protein expression and mRNA expression ($p < 0.001$) in TILs. LAG 3 expression was correlated with CD4+ and CD8+ T-cells within the tumor ($p < 0.001$). LAG3 expression showed an improved overall survival (OS) compared to patients without LAG3 expression (median OS 70.2 vs. 26.9 months; $p = 0.046$). The effect was even clearer in the group of patients with tumour stages > pT2 (70.2 vs 25.0 months; $p = 0.037$).

Conclusion This is the first description of LAG3 expression on TILs in EAC, underscoring the importance of immunomodulation in EAC. Our data suggest an impact of LAG3 in a relevant subset of EAC. Therapeutic studies investigating the efficacy of LAG3 inhibition in EAC will also provide predictive evidence and relevance of the immunohistochemical determination of LAG3 expression.

Keywords LAG3 · Immunohistochemistry · mRNA in-situ · Oesophageal adenocarcinoma

Abbreviations

CI	Confidence interval
EAC	Oesophageal adenocarcinoma
IgSF	Immunoglobulin superfamily
IHC	Immunohistochemistry
LAG3	Lymphocyte-activation-protein 3
MHC	Major histocompatibility complex
OS	Overall survival

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PD-1 Programmed death cell protein 1
TIL Tumour infiltrating lymphocyte
TMA Tissue microarrays

Introduction

Immunotherapy has grown to a rapidly advancing sphere of research on modern strategies for the treatment of cancer (Fridman 2017). A number of patients have already benefited from immune checkpoint blockades, and various drugs are currently under evaluation in clinical trials (Zhao and Subramanian 2018). In oesophageal adenocarcinoma (EAC)—a rapidly increasing cancer entity with a worse prognosis—surgery, chemotherapy and radiation remain the basis of treatment of EAC (Gore 2005; Grierson et al. 2017). The role of evolving immunotherapy has yet to be examined for EAC.

Immune checkpoints are a well-known form of immunomodulation, leading to a down-regulated immune response in the tumour microenvironment. Several of these checkpoints have been detected, such as programmed death cell protein 1 (PD-1), which is common and has occurred during the treatment of melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma and urothelial carcinoma (Andrews 2017; Roberts 2017; Ma 2017). In addition to PD-1, lymphocyte activation protein-3 (LAG3) presents a targetable checkpoint, as can contribute to therapy strategies, including treatment of EAC.

LAG3 belongs to the immunoglobulin superfamily (IgSF) and is particularly displayed on several forms of T-lymphocytes (CD4+, CD8+, regulatory T-cells [Treg], tumour infiltrating lymphocytes [TILs]), as well as B-lymphocytes and dendritic cells (He 2016). LAG3 shares approximately 20% identity with the CD4 gene, and it binds to major histocompatibility complex 2 (MHC II) with greater affinity than CD4 (He 2016). The LAG3/MHC II complex on CD4+ cells negatively modulates T-cell activity and enhances antigen self-tolerance when displayed on CD8+ cells. Conversely, LAG3 binding to MHC II on Treg cells advances the suppressive effect on T-lymphocytes, enforcing the negative immune regulation effect of LAG3 (Andrews 2017). Studies have suggested that LAG3 is a negative regulator of T-cell activation and function, since the blockade of LAG3 on human CD4 clones resulted in enhanced proliferation, with an elevated production of IL-2, IL-4, IFN-γ and TNFα (Previte, et al. 2019; Goldberg and Drake 2011).

Analysis of LAG3 overexpression on TILs has revealed evidence for a pathological role, which involves down-regulating the immune response for various cancer entities, such as chronic lymphatic leukaemia, colorectal cancer, ovarian cancer, melanoma and hepatocellular carcinoma, leading to a worse prognosis in LAG3 positive malignancies (Shapiro 2017; Li 2013; Hemon 2011; Huang 2015; Chen and Chen 2014). Interestingly, and in contrast to these findings, recent studies in breast cancer patients showed a favourable outcome in LAG3 positive tumours regarding the overall survival (OS) of the patients, while other studies showed a worse prognosis in breast cancer (Sidaway 2017; Burugu 2017).

The aim of the present study is to assess the expression of LAG3 on TILs at the protein level, as well as the mRNA level, in EAC and correlate the expression profile with clinico-pathological and molecular data and the prognosis of individual patients.

Material and methods

Patients and tumour samples

The formalin-fixed and paraffin embedded samples from 477 patients with EACs, who underwent primary surgical resection or resection after neoadjuvant therapy between 1999–2014 at the Department of General, Visceral and Cancer Surgery, University of Cologne, Germany, were analysed. The standard surgical procedures were laparotomic or laparoscopic gastrolysis and right transthoracic en-bloc oesophagectomy, with intrathoracic oesophagogastrostomy, including two-field lymphadenectomy of mediastinal and abdominal lymph nodes, transhiatal extended distal oesophagectomy with transabdominal intrathoracic or cervical anastomosis as described previously (Holscher 2007). Patients with advanced oesophageal cancer (cT3), or lymph node metastasis in clinical staging, received preoperative chemoradiation (5-Fluouracil, cisplatin, 40 Gy) or chemotherapy. Follow-up data were available for all patients. Patient characteristics are given in Table 1. Depending on the effect of neoadjuvant chemo- or radiochemotherapy, there was a preponderance of minor responders, defined as having a histopathological residual tumour of ≥ 10% (Schneider 2008).

According to the suggestions of the international immuno-oncology working group for assessing TILs on solid tumours, we constructed a multi-spot tissue microarray (TMA) (Simon et al. 2005; Helbig 2016; Hendry et al. 2017a, b). Construction of the multi-spot TMA and immunohistochemical staining procedures were performed as previously described (Simon et al. 2005; Helbig 2016). In brief, tissue cylinders, with a diameter of 1.2 mm each, were punched from selected tumour tissue blocks using a self-constructed, semi-automated precision instrument and embedded in empty recipient paraffin blocks. For the multi-spot TMA (165 patients), up to 8 tumour spots were punched out of the tumour, 4 spots each from the surface and the invasion front. The 165 patients evaluated using the multi-spot

Table 1 Baseline characteristics of the entire patient cohort

	Total	Percent (%)
Sex		
Female	42	10.0
Male	379	90.0
Age group		
≤ 65	221	52.4
> 65	200	47.6
Tumour stage		
pT 1	50	11.9
pT 2	36	8.6
pT 3	322	76.8
pT 4	11	2.6
Lymph node status		
pN 0	161	38.3
pN +	259	61.7

TMA were used as a test cohort. These data were statistically correlated with survival and molecular data, such as *TP53* mutational status.

We analysed 312 additional patients using a single-spot TMA. For this TMA, one tissue core from each tumour was randomly punched out and transferred into a TMA recipient block. 4 µm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ) for immunohistochemistry (IHC). All procedures performed for studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration, and its later amendments, or comparable ethical standards.

Immunohistochemistry

IHC was performed on the TMA slides. The following antibodies were used for IHC studies: a rabbit IgG monoclonal antibody (clone D2G40; dilution 1:300; Cell Signaling Technology, Netherlands) was used for LAG3, a rabbit monoclonal antibody (clone SP7; dilution 1:50; Thermo Fisher Scientific, USA) was used for CD3 and a mouse monoclonal antibody (clone C8/144B, dilution 1:200; Dako/Agilent, USA) was used for CD8. All immunohistochemical staining was performed using the Leica BOND-MAX stainer (Leica Biosystems, Germany) according to the protocol of the manufacturer. The evaluation of immunohistochemical expression was assessed manually by two pathologists (AQ and HL). Discrepancies in the results, which occurred only in a small number of samples, were resolved by consensus review.

Multicolour immunohistochemical stainings were performed on a Ventana Discovery Ultra automatic staining system (Ventana/Roche, Basel, Switzerland) using following primary antibodies: rabbit anti-LAG3 IgG monoclonal antibody D2G40, mouse anti-CD8 monoclonal antibody C8/144B, mouse anti-FOXP3 monoclonal antibody 236A/E7 (Abcam, UK; dilution 1:100), rabbit anti-CD4 monoclonal antibody 4B12 (Roche, Switzerland, ready to use). After conjugation with an antibody-bound enzyme (horseradish peroxidase or alcalic phosphatase), detection was carried out using DISCOVERY Silver kit (LAG3), DISCOVERY Yellow kit (FOXP3), DISCOVERY Teal kit (CD8), DISCOVERY Red Kit (CD4; all Ventana/Roche, Switzerland)). Counterstaining was done with hematoxylin and bluing reagent.

Strategy of evaluation

LAG3: < 1% of lymphocytes was defined as negative, 1–2% of lymphocytes were assessed as "LAG3 low", > 2% of lymphocytes was counted as "LAG3 high". The reading strategy followed the assessment of LAG3 in clinical trials in malignant melanoma, where the response rates of the LAG3 blockade correlated with LAG3 expression of ≥ 1% (Ascierto and McArthur 2017). For statistical analysis, the cut off was determined as ≥ 1%, thus low and high LAG3 expression was assessed as positive and < 1% expression as negative.

CD3: CD3 expression in < 3 lymphocytes/mm^2 was evaluated as negative, > 3–50 lymphocytes/mm^2 were assessed as low positive and > 50 lymphocytes/ mm^2 were defined as high positive, considering peritumoral and intratumoral distribution.

CD8: CD8 was analysed according to the CD3 evaluation criteria. For statistical analysis, high expression of CD3 or CD8 with > 50 lymphocytes/mm^2 were assessed as positive.

Regarding the multi-spot TMA considering eight tumor spots in total, four spots each of the tumour surface and the invasive margin, were examined. We calculated the average of the scores and matched the four samples to one category based on limit values: 0, negative; 0–0.9, low; 1–2, high (e.g. LAG3 expression in spot 1 = 2, spot 2 = 1, spot 3 = 0, spot 4 = 2, average of the spots: 1.25 → category "high"). Discrepancies in the results were resolved by consensus review.

Immunofluorescence multi-colour staining

Immunofluorescence staining was performed on TMAs and whole section slides. Therefore, paraffin sections were deparaffinised and antigens were retrieved with EDTA at pH 8 (PT Module, Lab Vision Thermo Scientific). Slides were blocked using normal horse serum, for 30 min at room temperature (Vector Laboratories). Slides were incubated overnight at 4 °C with a master mix containing the primary antibodies (LAG3, 1:75, Cell Signaling; CD4, mouse monoclonal 4B12, 1:75, Thermo Fisher Scientific; CD3, rat monoclonal CD3-12, 1:50, Abcam; CD8, 1:100, Dako/Agilent). Slides were washed and stained with a master mix containing the corresponding secondary antibodies coupled

to Alexa Fluor 555 (donkey anti-rabbit, Abcam), Alexa Fluor 594 (donkey anti-rat, Jackson Laboratories) and Alexa Fluor 647 (donkey anti-mouse, Jackson Laboratories) for 1 h at room temperature. Nuclei were visualised with DAPI (Sigma-Aldrich). Slides were mounted using an antifade solution (ProLong Diamond, Invitrogen) and scanned with a 40× objective (gSTED super-resolution confocal microscope, Leica). Images were adjusted for brightness and contrast using ImageJ (FIJI).

mRNA in-situ (RNAScope)

The RNAscope assay was performed according to manufacturer's instructions (Bott et al. 2011). In brief, paraffin-embedded TMA blocks were cut into 5 µm sections, pre-treated according to an extended protocol (30 min for pre-treatment 2 and 3), digested and hybridised at 40 °C in the HybEZ oven with the human LAG3 mRNA probe, which was provided by Advanced Cell Diagnostics Europe. The samples were then incubation with haematoxylin for 10 s. Target expression was compared to both negative (dapB) and positive (PPIB) controls. The scoring of the signals was performed as recommend by the manufacturer, where no staining or less than one molecule per 10 cells, score 0; 1–3 dots/cell, score 1; 4–9 dots/cell, score 2; 10–15 dots/cell, score 3; > 15 dots/cell, score 4. The DapB score was 0 and the PPIB score was 2. Positivity was defined as a score > 0. The determination of protein expression using immunohistochemistry, as well as mRNA expression, for LAG3 was assessed independently.

Analysis of TP53 mutation status

The TP53 status was evaluated by immunohistochemistry on the single-spot TMA. The results were correlated with the *TP53*-mutational status by parallel genomic sequencing. A detailed description of the analysis was described previously (Quaas 2019). In brief, tumor DNA extraction was followed by amplification with a customized GeneRead DNAseq Targeted Panel V2 (Qiagen, Hilden, Germany), library construction and quantification. Exons 5-8 of the *TP53 gene* were sequenced on the MiSeq (illumina, Berlin, Germany) with a variance-cutoff of 5%. The results were only interpreted if the coverage was > 200×.

Statistical analysis

Clinical data were collected prospectively according to a standardised protocol. SPSS Statistics for Mac (Version 21, SPSS) was used for statistical analysis. Interdependence between staining and clinical data were calculated using the chi-squared and Fisher's exact tests and displayed by cross-tables. Survival curves were plotted using the Kaplan–Meier method and analysed using the log-rank test. Univariate and multivariate analyses were performed for prognostic factors of overall survival using the Cox regression model. All tests were two-sided. p values < 0.05 were considered statistically significant.

Results

Patient baseline characteristics

Patient characteristics are given in Table 1. A total of 421 patients with EAC that underwent surgical tumour resection were immunohistochemically interpretable. Reasons for non-informative cases included lack of tissue samples or absence of unequivocal cancer tissue in the TMA spot. Operative procedures were either thoraco-abdominal en-bloc oesophagectomy ($n = 269$, 63.9%) with intrathoracic anastomosis or transhiatal oesophagectomy with transabdominal or cervical anastomosis ($n = 152$, 36.1%). For the single-spot TMA, 42 patients (10.0%) were female, 379 (90.0%) male. A similar distribution was found for the multi-spot TMA (9.7% female, 90.3% male). The median age of the entire patient cohort was 65.2 years (range 33.6–85.6 years) at the time of diagnosis. Neoadjuvant treatment (chemo- or radiochemotherapy) was administered to 271 patients (59.8%) before operation from the single-spot TMA samples and 23 patients (13.9%) from the multi-spot-TMA samples. The median follow-up for the entire cohort was 52.0 months.

LAG3 protein and mRNA expression

LAG3 immunostaining was localised in the cytoplasm/membrane of tumour infiltrating lymphocytes (Fig. 1). LAG3 expression of ≥ 1% was assessed as positive. In total, on the single-spot TMA, LAG3 positivity was detectable in 11.4% ($n = 48$) of interpretable EAC cases and 10.5% ($n = 17$) of the multi-spot TMA samples (Table 2). The latter demonstrated a heterogenic LAG3 distribution within the four spots of each patient, the invasive and the surface tumour margin, in 60.1% ($n = 100$). However, comparing the expression pattern of the surface with the invasive tumour margin, a low heterogeneity was observed, only one case (0.6%) was positive for LAG3 on the surface and not on the invasive margin.

LAG3 mRNA expression was analysed in 77 patients from the multi-spot TMA and positive in 36 patients (46.8%) on the surface margin and in 28 patients (35.9%) on the infiltration margin. Compared to detection by IHC, LAG3 mRNA expression showed higher expression frequencies on both the surface and infiltration margins. Despite the higher total number of LAG3 positive samples, as determined by mRNA base scope, there was a strong correlation between IHC and mRNA detection ($p < 0.001$). On the surface margin, 77.1% of the LAG3 IHC negative patients showed no mRNA expression, and similar results were found for the

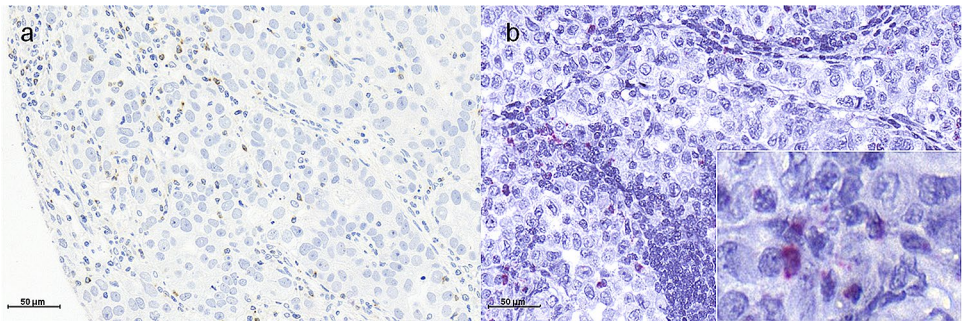

Fig. 1 Immunohistochemistry and mRNA-Scope analysis of LAG3 in EAC. **a** Immunohistochemical LAG3 expression on TILs; **b** LAG3 mRNA expression on TILs (red signals)

Table 2 Correlation of clinicopathological status and LAG3 expression of single-spot and multi-spot TMA

	LAG3 expression single-spot TMA						LAG3 expression multi-spot TMA					
		Negative		Positive		*p* value		Negative		Positive		*p* value
Total	421	373	88.6%	48	11.4%		161	140	84.8%	21	15.2%	
Sex												
Female	42	36	85.7%	6	14.3%	0.424	16	15	93.8%	1	6.3%	0.694
Male	379	337	88.9%	42	11.1%		145	125	86.2%	20	13.8%	
Age group												
< 65	221	189	85.7%	32	14.3%	0.546	70	65	92.9%	5	7.1%	0.060
> 65	200	179	89.4%	21	10.6%		90	74	82.2%	16	17.8%	
pT stage												
1	50	41	82.0%	9	18.0%	0.370	47	44	93.6%	3	6.4%	0.061
2	36	31	86.1%	5	13.9%		29	21	72.4%	8	27.6%	
3	322	290	90.1%	32	9.9%		82	72	87.8%	10	12.2%	
4	11	10	90.9%	1	9.1%		1	1	100.0%	0	0.0%	
pN stage												
0	161	135	83.9%	26	16.1%	0.018	61	55	90.2%	6	9.8%	0.352
pos	259	237	91.5%	22	8.5%		98	83	85.9%	15	14.1%	
Neoadjuvant therapy												
No	165	148	89.7%	17	10.3%	0.639	139	121	87.1%	18	12.9%	1.000
Yes	256	225	87.9%	31	12.1%		22	19	86.4%	3	13.6%	

infiltration margin (85.7%). No patient in any of the groups exhibited detectable protein expression without the presence of mRNA expression. No correlation between TP53 mutations, HER2 overexpression and LAG3 expression was revealed ($p = 0.383$ and $p = 1.000$, respectively).

CD3 and CD8

The status of CD3 was evaluated on single- and multi-spot TMA; CD8 infiltrating T-cells were only evaluated on the multi-spot TMA. CD8 demonstrated a similar distribution pattern as CD3, but to a lesser degree. High amounts of CD3 TILs were associated with an improved OS compared to CD3 poor tumours (Fig. 2a). In both tumour regions, roughly half of the tumours presented with a high accumulation of CD3 TILs (luminal 49.1%, invasive front 51.5%), which was correlated in the cross-table analysis ($p < 0.001$). There was no difference between the surface and the invasive tumour margin with respect to the amount of CD3 TILs. CD3 distribution within the tumour was predominantly peritumoral ($n = 130$; 78.8%) and showed no difference between the surface and the invasive tumour margin. High amounts of CD3 and CD8 TILs featured a strong correlation with high LAG3 expression ($p < 0.001$).

LAG3 and co-expression of T-cell subset markers

To evaluate which subtypes of T-cells expressed LAG3, we performed multicolour immunofluorescence and multicolour immunohistochemistry staining on two exemplary TMAs. In a semiquantitative analysis, we correlated LAG3 positive cases with the expression of CD3, CD4 and CD8 (Figs. 3 and 4). In normal lymphatic tissue, LAG3 is co-expressed with CD3, CD4 CD8 and FOXP3. In EAC multicolour immunohistochemistry, a predominant co-expression of LAG3 and CD8 was seen, only a minor fraction demonstrated positivity for LAG3 and CD4 or FOXP3.

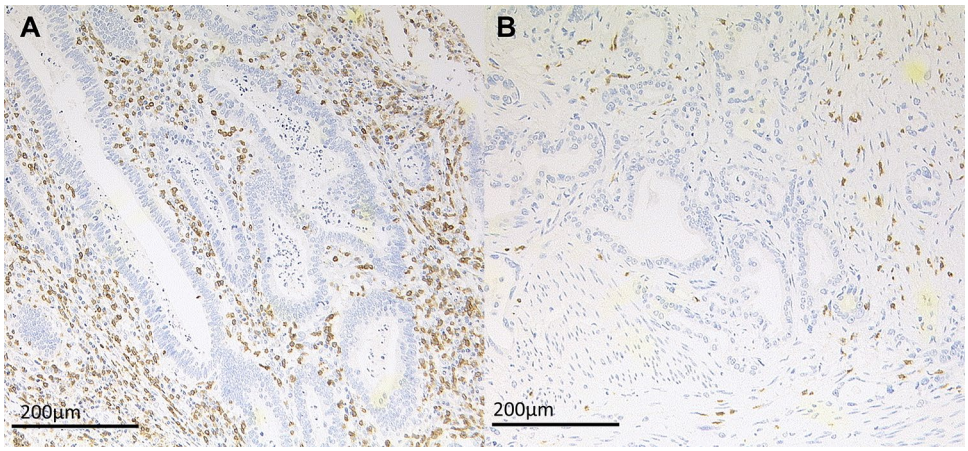

Fig. 2 Immunohistochemistry of CD3 in EAC. **a** High expression of CD3 with > 50 positive TILs/mm^2; **b** low expression of CD3 positive TILs

In multicolour immunofluorescence LAG3 positive cells within the tumour microenvironment were seen with co-expression of CD3, CD4 and CD8.

LAG3 as a prognostic biomarker

To analyse the impact of LAG3 expression on TILs, Kaplan Meier survival analysis were performed on the single-spot TMA. The OS in patients with LAG3 expression significantly improved compared to LAG3 negative tumours. The

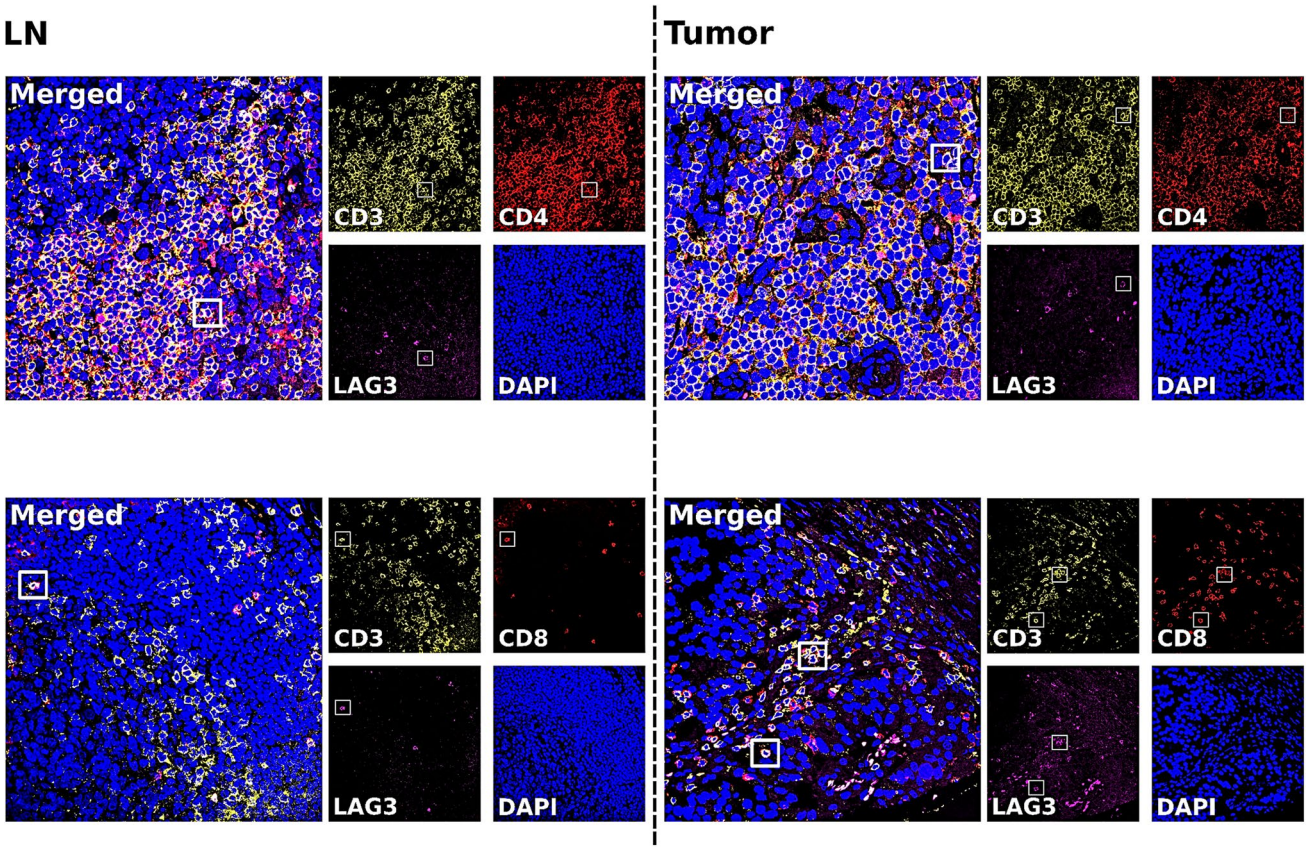

Fig. 3 Immunofluorescence of multicolour staining for LAG3 (purple signals), CD3 (yellow signals), CD4 and CD8 (red signals) and counterstaining of the nuclei with DAPI (blue signals). **a**, **b** Show normal lymph node tissue; in **a** with high expression of CD3 and CD4 and single-cell co-expression with LAG3; in **b** single cells positive for LAG3 co-expressed CD3 and CD8. **c**, **d** Show tumour tissue with surrounding immune cells; in **c** the co-expression of LAG3 with CD3 and CD4 is seen; in **d** LAG3 positive cells are positive for both CD3 and CD8

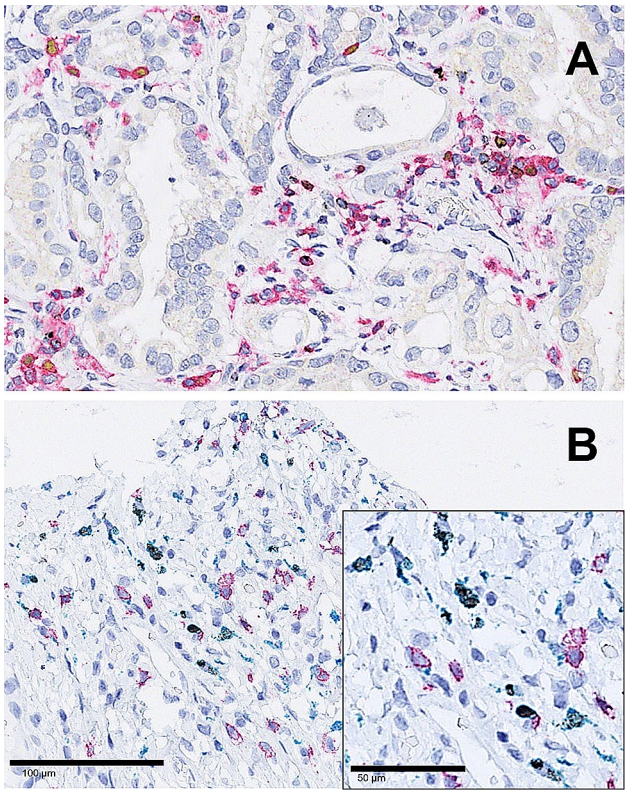

Fig. 4 Multicolour IHC for LAG3 (black signals), CD4 (red signals), FOXP3 (yellow signals) and CD8 (blue signals): **a** co-expression of LAG3, CD4 and FOXP3 in a minor fraction; **b** predominant co-expression of LAG3 with CD8 (inserted detail), here no relevant co-expression with CD4

median OS was 70.2 months (95% confidence interval (CI) 1.9–138.5 months) in LAG3 positive tumours compared to a median OS of 26.9 months (95% CI 21.9–31.8 months, $p = 0.046$) in LAG3 negative cases (Fig. 5a). The effect was independent of whether neoadjuvant treatment was administered or not. The observed survival difference in the entire patients´ cohort is predominantly driven by advanced tumour stages (> pT2). LAG3 positive tumours with tumour stages > pT2 had a median OS of 70.2 months (95% CI 20.6–32.0 months), while LAG3 negative tumours with tumour stages > pT2 showed a median OS of 25.0 months (95% CI 2.1–154.6 months) ($p = 0.037$). In the group of pT1/2 tumours, a LAG3 dependent survival difference could not be revealed (Fig. 5b and c). In a multivariate cox-regression model, LAG3 expression alone failed to serve as an independent prognostic marker due its correlation with advanced tumour stages (Table 3).

Discussion

In a large set of 421 patients with EAC, we report the impact of the checkpoint inhibitor LAG3 considering the protein and mRNA expression, as well as the distribution pattern within the tumour, in correlation with clinical and molecular data. In our cohort, LAG3 was positively correlated with subset of T-cells (predominant CD8 positive T-cells). Additionally, elevated LAG3 expression was linked to a significantly better outcome for patients in the advanced tumour stages subgroup.

The construction of the multi-spot TMA for the analysis of tumour heterogeneity and associated TILs followed the recommendations of the International Immuno-Oncology Biomarkers Working Group (Hendry et al. 2017a, b). We started our analysis with a test-cohort of 165 patients using the multi-spot TMA, followed by a much larger cohort considering additional patients to reassess the results found before. The multi-spot TMA demonstrated a heterogenic LAG3 distribution within the eight tumour spots; however, by comparing the tumour surface with the infiltration margin, we found a consistent expression pattern of LAG3.

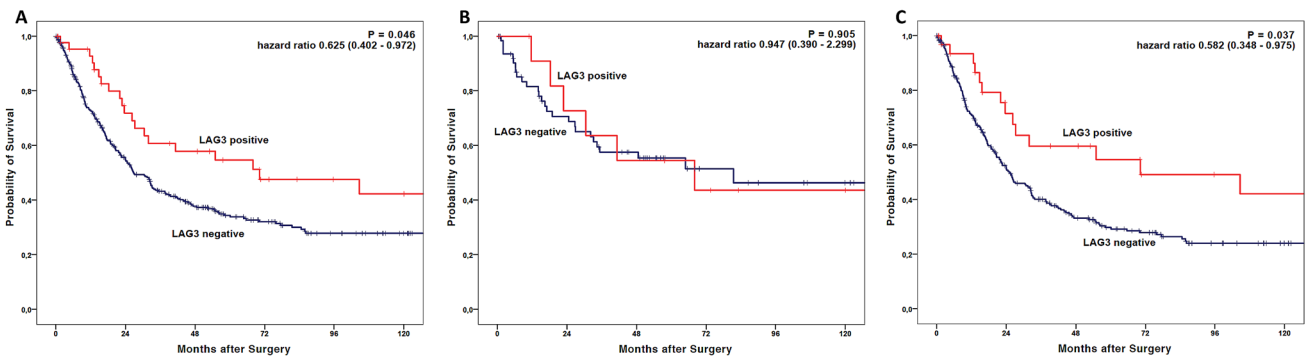

Fig. 5 **a** Tumours with LAG3 positive TILs showed a better overall survival. 70.2 months (95% confidence interval (CI) 1.9–138.5 months) in LAG3 positive tumours compared to a median OS of 26.9 months (95%CI 21.9–31.8 months, $p = 0.046$) for LAG3 negative tumours. **b** In early invasive tumours, LAG3 dependent survival difference could not be revealed, the survival difference in the entire patients´ cohort is therefore driven by advanced tumour stages (< pT2) (**c**)

Table 3 Multivariate analysis of clinico-pathological status and LAG3 expression for the entire patients´ cohort analysed on the single-spot TMA

	Significance (*p* value)	Hazard ratio	95% confidence interval	
			Lower	Upper
Sex (female vs. male)	0.023	1.742	1.081	2.808
Age group (< 65 vs > 65 years)	0.212	1.149	0.924	1.428
pT (pT1/2 vs pT3/4)	0.018	1.501	1.072	2.100
pN (pN0 vs pN +)	< 0.001	2.804	2.103	3.739
LAG3 (pos. vs neg.)	0.312	0.837	0.593	1.182

Thus, in the case of endoscopically taken biopsies LAG3 expression in EAC can serve as a reliable predictor regarding the overall LAG3 expression within the tumour.

In contrast to our initial hypothesis, we are able to show a favourable outcome of patients with elevated LAG3 in TILs. However, most probably due to a correlation with pT-stage and lymph node metastasis, the LAG3 expression failed to serve as an independent prognostic marker, since the survival benefit correlated with LAG3 expression was predominantly detectable in pT3/4 tumours. This is fully in line with recent studies on breast cancer patients but in contrast to previous results in other solid tumour entities, such as melanoma and hepatocellular carcinoma (Li 2013; Hemon 2011; Sidaway 2017). However, divergent descriptions concerning the prognostic impact of a single immune checkpoint between different tumour entities are well known and likewise given for PD-1/PD-L1 (Bertucci 2015, 2017). This phenomenon can be explained by various analysis techniques and antibodies or by different organic systems. Due to technical variabilities, we analysed the mRNA expression profile of LAG3 for 77 patients and compared it to the protein expression detected using IHC in a double-blinded examination. Since there was a distinct correlation between the mRNA and protein expression profiles of LAG3, we confirm the viability of our antibody and analysis techniques.

The favourable outcome in context with LAG3 expression appears to be counterintuitive, initially, considering the suppressive effect of this immune checkpoint. Furthermore, our distinct results require exploratory approaches to clarify the role of immunomodulation in EAC. We, therefore, developed hypotheses to explain how LAG3 overexpression could be related to a better outcome.

An elevated expression may be part of a powerful immune reaction leading to an effective antitumoral response. Thus, we assume that pro-inflammatory signalling pathways against the tumour could be compensatory regulation by the suppressive effect of LAG3. We found a strong correlation between the existence of T-cells (CD3) and LAG3, supposing that an inflammatory microenvironment is attended by increased LAG3. Since tumours with elevated T-cells showed a significantly better outcome in our cohort, we underline the importance of an inflammatory antitumoral response, which is in accordance with former studies on EAC (Noble 2016). We therefore presume that LAG3 serves as a biomarker for a strong immune response. The initially assumed inhibitory immunomodulatory effect does not seem to be reflected by our current analysis to the extent expected. As described above, similar effects have been demonstrated for PD-L1 expression in different tumour entities. Whether a pharmacological LAG3 inhibition will prove to be effective in EAC cannot be conclusively answered on the basis of the available data. In our opinion, however, a positive effect of LAG3 inhibitor therapy can be assumed. However, further functional investigations with regard to LAG3 expression and interactions with tumour cells are necessary.

Taken together, we presume that LAG3 serves as a surrogate parameter in immunogenic tumour biology; although, we must admit that the limited knowledge of downstream mechanisms and the interaction with other intracellular pathways only allows us to hypothesise about a possible function of the protein in EAC. In addition, it remains obscure why only advanced tumour stages revealed a prognostic impact of LAG3 in contrast to early stages, underlining the urgent need for further research on immunomodulation.

Nevertheless, our assumptions support the use of LAG3 inhibition for EAC, since the loss of regulatory survival mechanisms would lead to a stronger immune response via CD3/8. Therapeutic studies investigating the efficacy of LAG3 inhibition in EAC will also provide predictive evidence on the determination of the LAG3 expression in the tumour microenvironment, as we have learned in the past for the determination of PD-L1 in lung cancer.

Availability of data and material

The original data can be requested from the corresponding author, including SPSS tables and statistical syntax, immunohistochemical and rRNA data.

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Author contributions FG: study conception, statistical analysis, manuscript writing, operation of patients, immunohistochemistry analysis; MK: immunohistochemistry and mRNA analysis, manuscript writing; CB: manuscript writing, manuscript revision, operation of patients; HAS: manuscript revision, immunofluorescence analysis; MT: immunofluorescence analysis; PL: manuscript revision, immunohistochemistry staining; WS: manuscript revision, operation of patients; TZ: manuscript writing, data analysis; HA: data analysis, operation of patients: RB: mRNA analysis, manuscript revision; HL: manuscript writing, immunohistochemistry and mRNA analysis, TMA construction; AQ:

TMA construction, immunohistochemistry supervision, manuscript writing, manuscript revision.

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Compliance with ethical standards

Conflict of interest Reinhard Büttner: Co-Founder and Co-Owner of Targos Molecular Pathology Inc. Kassel/Germany. Hans Schlösser: Funding for Research from Astra Zeneca for research outside this study. Thomas Zander: BMS: advisory board, clinical trial; Novartis: advisory board, clinical trial; Lilly: advisory board, clinical trial; MSD: advisory board, clinical trial. The remaining authors declare no conflict of interest or other financial disclosures.

Ethics approval The study was approved by the local ethics committee, due to the anonymised character of the study, individual consent of the patients was not requested.

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Research Article

Indoleamine 2,3-Dioxygenase (IDO) Expression Is an Independent Prognostic Marker in Esophageal Adenocarcinoma

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Background. Indoleamine 2,3-dioxygenase (IDO) is an interferon-inducible immune checkpoint expressed on tumor-infiltrating lymphocytes (TILs). IDO is known as a poor prognostic marker in esophageal squamous cell cancer, while a positive effect was shown for breast cancer. A comprehensive analysis of IDO expression in a well-defined cohort of esophageal adenocarcinoma (EAC) is missing. *Methods*. We analyzed 551 patients with EAC using single-protein and multiplex immunohistochemistry as well as mRNA in situ technology for the expression and distribution of IDO on subtypes of TILs (INF-γ mRNA and CD4- and CD8-positive T lymphocytes). *Results*. IDO expression on TILs was seen in up to 59.6% of tumors, and expression on tumor cells was seen in 9.2%. We found a strong positive correlation of IDO-positive TILs, CD3-positive T lymphocytes, and INF-γ mRNA-producing TILs in the tumor microenvironment of EACs showing significantly better overall survival (47.7 vs. 22.7 months, $p < 0.001$) with emphasis on early tumor stages (pT1/2: 142.1 vs. 37.1 months, $p < 0.001$). In multivariate analysis, IDO is identified as an independent prognostic marker. *Conclusions*. Our study emphasizes the importance of immunomodulation in EAC marking IDO as a potential biomarker. Beyond this, IDO might indicate a subgroup of EAC with an explicit survival benefit.

1. Background

Esophageal cancer is associated with the sixth highest cancer-related mortality rate and a median survival time of 29 months [1, 2]. Although esophageal adenocarcinoma (EAC) is the fastest growing cancer in the western world, most of the therapy concepts for EAC remain largely ineffective [3]. Multimodal therapy consists of esophageal en bloc resection and perioperative radiochemotherapy; nevertheless, new therapeutic options are urgently needed to improve therapeutic concepts and prognosis in EAC.

The interaction of the tumor and its associated immune compartment is supposed to play an important role in cancer progression [4]. Mechanisms of immunosuppression in tumor microenvironment are not completely understood, although antigen loss and negative regulation by immune checkpoints are presumed to lead to dysfunction of T cells [5, 6]. Thus, identification of further immune-modulating targets is an important part of the current cancer research.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme affecting T cell activity and immune tolerance. IDO expression has been detected in immune cells, stromal cells, and cancer cells and revealed relevance in cancer development and progression [7]. The enzyme catalyzes the rate-limiting step in the catabolism of local tryptophan which finally leads to anergy of effector T cells and promotion of

regulatory T cells (Tregs) [8]. In several malignancies [9–11], IDO expression in either tumor cells or tumor-associated cells has been linked to adverse outcome, as demonstrated by Jia et al. for esophageal squamous cell carcinoma (ESCC) [12]. This is contrasting other studies with evidence for an improved survival in renal cell carcinoma and breast cancer [13].

The mechanism of IDO induction is not completely clear. However, it is widely accepted that interferons, particularly INF-γ, stimulate IDO expression in various cell types [14]. INF-γ is a pleiotropic cytokine supposed to play a central role in antitumor immunity with cytostatic, proapoptotic, and immune-provoking effects [15]. Besides the antitumoral effect, there is still evidence for an alternative, protumorigenic impact of γ-INF [16]. Interestingly, IDO induction via INF-γ is one mechanism presumed to contribute to an immunosuppressive tumor microenvironment in malignant melanoma and colorectal cancer [16, 17]. Recently, Rosenberg et al. analyzed the mRNA data of IDO and other checkpoint markers in The Cancer Genome Atlas (TCGA) cohort of esophageal cancer [18]. They found IDO to be related to worse prognosis in both squamous cell cancer and adenocarcinoma of the esophagus. However, both tumor entities included a relatively small number of patients (ESCC 87 and EAC 97), and according to EAC data, only gene expression analysis was performed. So, nothing is known about the protein expression of IDO in a clinical setting of neoadjuvant-treated or primary resected esophageal adenocarcinoma patients.

In the present retrospective study, we tested the hypothesis that the protein expression of the immune checkpoint IDO on immune cells is prognostic in a large cohort of EAC. Expression levels and spatial distribution of IDO, INF-γ and CD3 in the tumor microenvironment were therefore analyzed using immunohistochemistry and RNA BaseScope technology on tissue microarrays (TMAs) in two cohorts. Protein distribution on the different immune cells and heterogeneity were considered, and results were correlated with clinical and molecular data.

2. Material and Methods

2.1. Patients and Tumor Samples. We analyzed formalin-fixed and paraffin-embedded (FFPE) material of 551 patients with esophageal adenocarcinomas that underwent primary surgical resection or resection after neoadjuvant therapy between 1999 and 2015 at the Department of General, Visceral and Cancer Surgery, University of Cologne, Germany. The standard surgical procedure consisted of a transthoracic en bloc esophagectomy with two-field lymphadenectomy (abdominal and mediastinal lymph nodes); reconstruction was done by formation of a gastric tube with intrathoracic esophagogastrostomy (Ivor-Lewis esophagectomy) [19]. The abdominal phase was predominantly performed as a laparoscopic procedure (hybrid Ivor-Lewis esophagectomy). Technical details of this operation are described elsewhere [20–22]. Patients with advanced esophageal cancer (cT3) or presence of lymph node metastasis in clinical staging received preoperative chemoradiation (5-Fluouracil, cisplatin, 40 Gy) or chemotherapy alone. Follow-up data were available for all patients. Patient characteristics are given in Tables 1 and 2. Depending on the effect of neoadjuvant chemo- or radiochemotherapy, there is a preponderance of minor responders, defined as histopathological residual tumor of ≥10% [23]. This retrospective study was performed in accordance with the ethical standards of the ethics committee of the University of Cologne and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The recently published criteria for reporting recommendations for tumor marker prognostic studies (REMARK criteria) were followed in this study [24, 25].

2.2. Tissue Microarray (TMA). Immunohistochemistry and RNA BaseScope analyses were performed on tissue microarrays. Construction of the TMA was described previously [26, 27]. In brief, tissue cylinders with a diameter of 1.2 mm each were punched out from selected tumor tissue blocks using a self-constructed semiautomated precision instrument and embedded in empty recipient paraffin blocks.

In the first step, we analyzed a test cohort of 165 EAC. Therefore, we built a tissue microarray (TMA) with multiple tumor spots according to the suggestions of the international immunooncology working group for assessing tumor-infiltrating lymphocytes (TILs) on solid tumors [26–29]. Up to 8 tumor spots were punched out of the tumor considering the surface and the invasion front. In the second step, we analyzed 386 additional patients to confirm our results using a single-spot TMA.

The immunohistochemical and RNA BaseScope data were statistically correlated with survival and molecular data like *TP53* mutational status and HER2/neu status.

2.3. Immunohistochemistry. Immunohistochemistry (IHC) was performed on TMA slides. For IDO the rabbit IgG monoclonal antibody (D5J4E; dilution 1 : 400; Cell Signaling Technology, USA) and for CD3 the rabbit monoclonal antibody (SP7; dilution 1 : 50; Thermo Fisher Scientific, USA) were used. All immunohistochemical stainings were performed using the Leica BOND-MAX stainer (Leica Biosystems, Germany) according to the protocol of the manufacturers. The evaluation of immunohistochemical expression was assessed manually by two pathologists independently (PL and HL). Discrepant results were resolved by consensus review.

2.4. Strategy of Evaluation. For IDO, the expression in <1% lymphocytes was defined as negative (score = 0), 1-4% of positive lymphocytes was assessed as "low positivity" (score = 1), and >4% of lymphocytes was counted as "highly positive" (score = 2). For statistical analysis, IDO-negative tumors were tested against IDO (low and high)-positive tumors.

For CD3, CD3 expression in <3 lymphocytes/mm^2 was evaluated as negative, >3–50 lymphocytes/mm^2 were assessed as low positive, and >50 lymphocytes/mm^2 were defined as highly positive considering peritumoral and intratumoral distribution.

Concerning the multispot TMA, four spots of tumor surface and invasive margin each were examined. We built the

Table 1: Patient characteristics and IDO expression results on the test cohort ($n = 165$; 159 analyzable).

			IDO expression surface margin					IDO expression infiltration margin				
			Negative		Positive		p value	Negative		Positive		p value
	No.	%	No.	%	No.	%		No.	%	No.	%	
Sex												
Female	16	10.1%	5	31.3%	11	68.7%	0.430	5	33.3%	10	66.7%	0.783
Male	143	89.9%	62	43.4%	81	56.6%		58	41.1%	83	58.9%	
Age group												
<65 years	70	44.3%	31	44.3%	39	55.7%	0.746	31	45.6%	37	54.4%	0.248
>65 years	88	55.7%	36	77.0%	52	23.0%		31	35.6%	56	64.4%	
Tumor stage												
pT1	46	29.1%	13	28.3%	33	71.7%	0.070	13	29.5%	31	70.5%	0.200
pT2	29	18.4%	12	41.4%	27	58.6%		11	37.9%	18	62.1%	
pT3	82	51.9%	41	50.0%	41	50.0%		37	45.7%	44	54.3%	
pT4	1	0.6%	1	100%	0	0.0%		1	100%	0	0.0%	
Lymph node metastasis												
pN0	60	38.0	17	23.3%	43	71.75	0.022	20	33.9%	39	66.1%	0.154
pN1	71	44.9	34	47.9%	37	52.1%		26	37.0%	43	62.3%	
pN2	12	7.6	6	50.0%	6	50.0%		7	58.3%	5	41.7%	
pN3	15	9.55	10	66.6%	5	33.3%		9	60.0%	6	40.0%	
UICC stage												
I	41	26.1%	21	51.2%	20	48.8%	<0.001	23	57.6%	17	42.5%	0.045
II	21	13.4%	15	71.4%	6	28.6%		14	66.7%	7	33.3%	
III	75	47.8%	69	92.0%	6	8.0%		60	78.9%	16	21.1%	
IV	20	12.7%	17	85.9%	3	15.0%		17	85.0%	3	15.0%	

average of the scores and matched the four samples to one category based on limit values: 0-0.49 = negative and 0.5-2 = positive.

2.5. Multiplex Immunohistochemistry. Multiplex immunohistochemistry staining was performed on a Ventana Discovery Ultra automatic staining system using TMA slides. The following primary monoclonal antibodies were used: IDO, Cell Signaling; mouse CD8 clone C8/144B, mouse CD68 clone PG-M1 (both Dako/Agilent, USA), and rabbit CD4 clone 4B12 (Roche, Switzerland, ready to use). After conjugation with an antibody-bound enzyme (horseradish peroxidase or alcalic phosphatase), detection was carried out using DISCOVERY Silver kit (IDO), DISCOVERY Yellow kit (CD68), DISCOVERY Teal kit (CD4), and DISCOVERY Purple Kit (CD8; all Ventana/Roche, Switzerland). Counterstaining was done with hematoxylin and bluing reagent.

2.6. RNA In Situ (RNA BaseScope). The RNA BaseScope assay was performed according to the manufacturer's instruction. In brief, paraffin-embedded TMA blocks were cut into 5 μm sections, pretreated according to extended protocol (30 minutes for pretreatments 2 and 3), digested, and hybridized at 40°C in the HybEZ oven with human INF-γ mRNA probe provided by Advanced Cell Diagnostics (ACD, United Kingdom, Europe). Incubation time with hematoxylin was 10 seconds. Target expression was compared to both negative (dapB) and positive (PPIB) controls. Scoring of signals was done as recommend by the manufacturer with no staining or less than one molecule per 10 cells (TILs) = score 0, 1-3 dots/cell = score 1, 4-9 dots/cell = score 2, 10-15 dots/cell = score 3, and >15 dots/cell = score 4. dapB score was 0 and PPIB score was 2. Positivity was defined as a score > 0.

2.7. Statistical Analysis. Clinical data were collected prospectively according to a standardized protocol. SPSS Statistics for Mac (Version 21, SPSS) was used for statistical analysis. Interdependence between stainings and clinical data were calculated using the chi-squared and Fisher's exact tests and displayed by cross-tables. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Univariate and multivariate analyses were performed for prognostic factors of overall survival using the Cox regression model. All tests were two sided. p values < 0.05 were considered statistically significant.

3. Results

3.1. Clinicopathological Characteristics. The test cohort comprised 165 patients with EAC that underwent surgical resection. There was a male preponderance with 149 male (90.4%) and 16 female (9.6%) patients with a median age of 65.1 years (range 33-85 years) at the time of operation. To confirm our results, a single-spot TMA with additional 386 patients was analyzed, resulting in 551 patients in total. The median follow-up for the entire cohort was 57.7 months with a calculated 5-year survival rate of 26.6%. 159 (96.3%) of the

Table 2: Patient characteristics of the entire cohort, IDO expression results ($n = 551$; 496 patients analyzable).

	IDO expression single spot				p value
	Negative		Positive		
	No.	%	No.	%	
Sex					
Female	31	53.4%	27	46.6%	0.331
Male	204	46.6%	234	53.4%	
Age group					
<65 years	132	51.0%	127	49.0%	0.166
>65 years	105	44.5%	132	55.5%	
pT1	13	26.8%	52	73.2%	
pT2	27	49.1%	28	50.9%	0.004
pT3	179	51.1%	171	48.9%	
pT4	9	50.0%	9	50.0%	
pN0	74	38.1%	120	61.9%	
pN1	79	44.6%	98	55.4%	<0.001
pN2	40	64.5%	22	35.5%	
pN3	40	65.6%	21	34.4%	
Neoadjuvant treatment					
Yes	90	41.7%	126	58.3%	0.029
No	145	51.8%	135	48.2%	
UICC stage					
I	34	33.7%	67	66.3%	
II	44	38.9%	69	61.1%	<0.001
III	118	57.3%	88	42.7%	
IV	37	50.0%	37	50.0%	

samples were evaluable (Table 1) in the test cohort and 496 samples (90.0%) in the complete cohort (Table 2). The reason for noninformative cases was the absence of unequivocal cancer tissue on the TMA spot. Figure 1 shows the survival data correlated with UICC stage in EAC.

3.2. Immunohistochemical Analysis of IDO. IDO immunostaining was localized in the cytoplasm/membrane of tumor-infiltrating lymphocytes and cancer cells (Figures 2(a) and 2(b)). In the test cohort, IDO expression on TILs (low and high positivity) was seen in 57.9% ($n = 92$) on the surface margin and 59.6% ($n = 93$) on the infiltration margin with a high correlation between the two localizations (surface and infiltration; $p < 0.001$). Cross-table analysis revealed a correlation between IDO expression and nodal-negative patients ($p = 0.022$) and low UICC stages (UICC I/II, $p = 0.004$).

On the single-spot TMA, we found IDO expression on TILs in 261 patients (52.6%). Again, a strong correlation between IDO-positive samples and early tumor stages (pT1/2) ($p = 0.022$) as well as nodal-negative patients ($p = 0.012$) was seen. IDO expression was detectable on cancer cells in 63 patients (9.2%) without any correlation with clinicopathological data.

3.3. Multiplex Immunohistochemistry for Subtyping of T Cells. To evaluate which subtypes of T cells expressed IDO, we performed multiplex immunohistochemistry staining on two exemplary TMAs. We correlated IDO-positive cases with the expression of CD4, CD8, and CD68 semiquantitatively (Figure 3). For IDO, a predominant coexpression with CD4 was seen, and a minor fraction demonstrated positivity for CD68. A coexpression with CD8 was not reliably detectable.

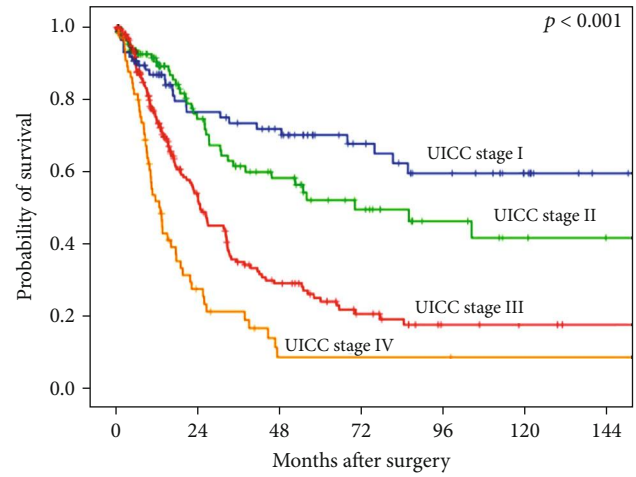


Figure 1: UICC stage adjusted survival for the entire patient cohort ($n = 551$).

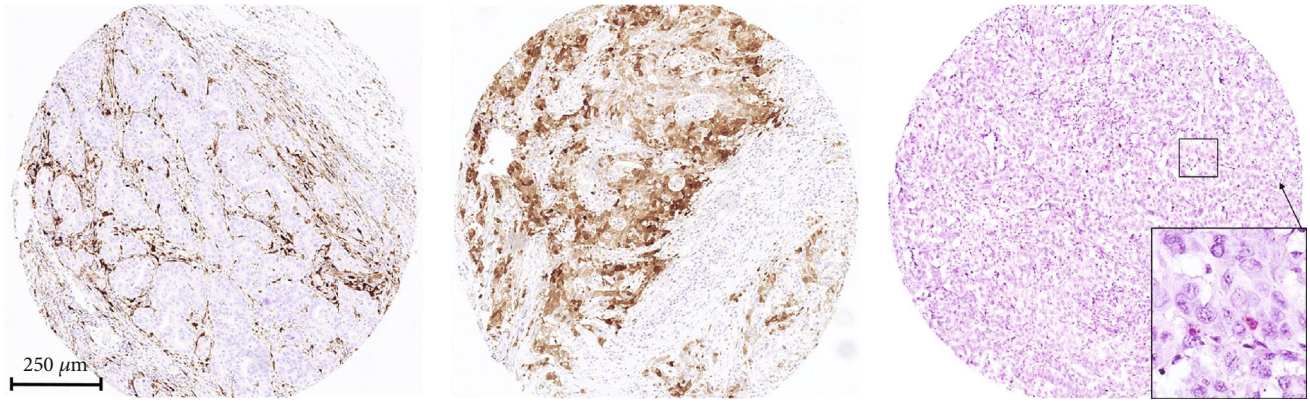
3.4. Immunohistochemical Analysis of CD3. The expression of CD3 was evaluated for multispot (test cohort) and single-spot TMA. CD3 distribution was predominantly seen peritumorally ($n = 130$; 78.8%). In the test cohort, roughly half of the tumors presented with high levels of CD3 (tumor surface 49.1%, infiltration margin 51.5%), which correlated well in cross-table analysis ($p < 0.001$). There was no difference between surface and infiltration margins with respect to the amounts of CD3-positive TILs.

IDO expression on TILs positively correlated with the amount CD3-positive T cells within the tumor ($p < 0.001$).

High levels of CD3-positive TILs are associated with an improved overall survival (OS) compared to CD3-poor tumors considering the single-spot TMA of 551 patients (496 patients analyzable; $p = 0.002$; Figure 4(a)).

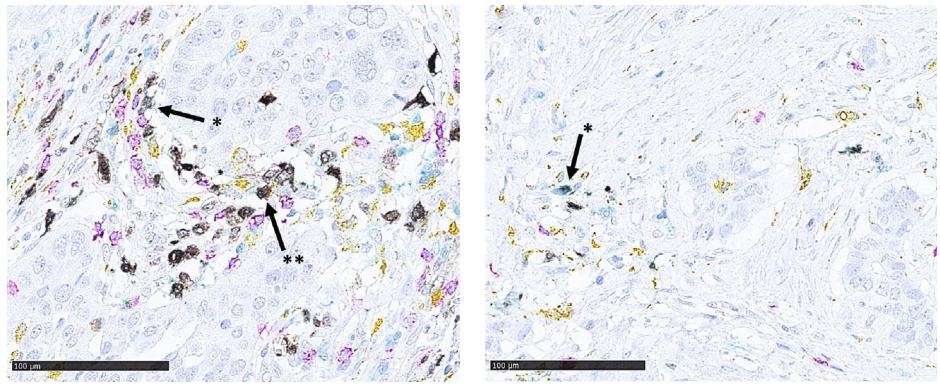
3.5. RNA BaseScope Analysis of INF-γ. Within the test cohort, correlation between INF-γ and IDO-positive TILs revealed a strong correlation in both compartments, surface and infiltration zones, respectively ($p < 0.0001$) (Figure 2(b)).

3.6. Correlation with Molecular Markers. TP53 mutation and HER2 amplification/expression status was available for 356 patients. There was no correlation between the remaining histopathological parameter and *TP53* mutational or HER2/neu status. Within the IDO-positive group, 117 patients showed a *TP53* mutation (60.6%) and 76 patients (39.6%) were *TP53* wild-type tumors ($p = 0.210$). Similar results were found for HER2/neu amplification. In tumors with high IDO expression, 26 patients showed HER2/neu amplification (13.8%), but a correlation via cross-table analysis did not



(a) (b) (c)

Figure 2: Immunohistochemistry of IDO and INF-γ mRNA analysis: (a) high IDO expression on tumor-infiltrating lymphocytes; (b) IDO expression of tumor cells; (c) mRNA of INF-γ (red signals) on tumor cells.



(a) (b)

Figure 3: Multicolor immunohistochemistry for IDO (black signals), CD4 (teal/blue signals), CD8 (purple signals), and CD68 (yellow signals): (a) coexpression of IDO and CD4 (∗) and CD68 (∗∗); (b) coexpression with CD4 (∗).

reveal a significant association between IDO and HER2/neu amplification ($p = 0.116$).

3.7. Expression of IDO on TILs Is Prognostic in EAC. In the test cohort of 165 patients, no statistically significant overall survival difference was detectable for IDO-expressing tumors, although a correlation with nodal-negative tumors and low UICC stages in IDO-positive samples was seen. However, there was a trend towards improved overall survival (OS) in patients with IDO expression.

On the single-spot TMA, tumors with IDO-expressing TILs showed an improved median OS (47.7 months (95% confidence interval (CI) 20.9-73.8 months)) compared to IDO-negative patients (median OS 22.7 months (95% CI 18.8-26.6 months), $p \leq 0.001$) (Figure 4(b)). The hazard ratio was 0.581 (95% CI 0.440-0.767, $p < 0.001$) for patients with IDO expression.

Subgroup analyses revealed a particularly pronounced difference in OS in the group of pT1/2 stage patients. Within this group of lower tumor stages, IDO-positive patients reached a calculated average OS of 142.1 months (median not reached, average 95% CI 115.2-168.9 months) compared to an average OS of 37.1 months (95% CI 23.6-50.7 months, median OS 30.5 months (95% CI 19.9-41.1 months, $p < 0.001$)) (Figure 4(c)). However, the survival difference remains significant also in the subgroup of higher tumor stages (pT3/4) with comparable median OS values to the entire patient cohort. In the pT3/4 group, the median OS for IDO-positive patients was 33.3 months (95% CI 17.9-48.7 months) and 22.1 months (95% CI 17.9-26.3 months, $p = 0.035$) for IDO-negative patients (Figure 4(d)).

In a multivariate Cox regression analysis, IDO expression on TILs and the histopathological parameters pT and pN stages were independent prognostic markers (Table 3).

3.8. Impact of Neoadjuvant Therapy. 216 (43.5%) patients received neoadjuvant therapy, whereas 280 (56.4%) patients primarily underwent surgical resection. The prognostic impact of IDO on OS was independent on whether neoadjuvant treatment was administered or not. IDO expression remains a positive prognostic marker in both patient groups with primary surgery and surgery with neoadjuvant treatment. Patients who underwent primary surgery without any kind of neoadjuvant treatment with the presence of IDO expression showed a median OS of 104.6 months (95% CI 50.1–159.2 months) while IDO-negative patients showed an

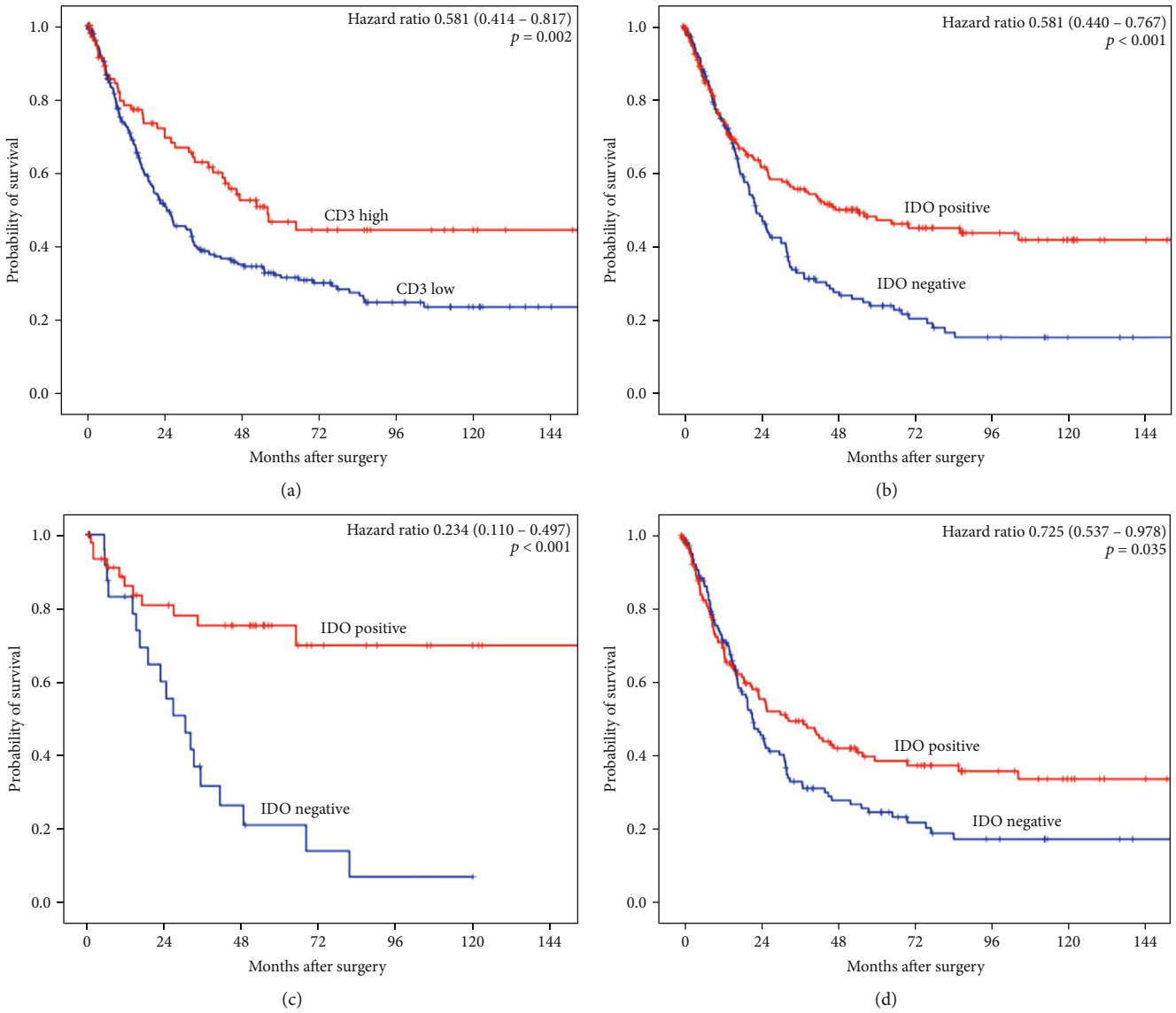


Figure 4: (a) High amounts of CD3-positive T cells are associated with an improved OS in esophageal adenocarcinoma. (b) Tumors with IDO-positive TILs show better median overall survival of 47.7 months in IDO-positive tumors compared to a median OS of 22.7 months for IDO-negative tumors. (c) In the pT1/2 group, patients with IDO-positive expression have a calculated average OS of 142.1 months (median not reached) compared to an average OS of 37.1 months (median OS 30.5 months), $p < 0.001$. (d) The survival difference remains significant also in the subgroup of pT3/4 tumor stages with median OS of 33.3 months for IDO-positive tumors and 22.1 months for IDO-negative tumors ($p = 0.035$).

Table 3: Multivariate Cox regression model; HR = hazard ratio.

	Hazard ratio	95% confidence interval		p value
		Lower	Upper	
Sex (male vs. female)	1.557	0.863	2.807	0.141
Age group (<65 vs. >65 years)	1.351	1.01	1.807	0.043
Tumor stage (pT1/2 vs. pT3/4)	1.429	0.916	2.229	0.116
Lymph node metastasis (pN0 vs. pN+)	2.987	2.105	4.239	0.022
CD3 (low vs. high)	0.666	0.459	0.966	0.032
IDO on TILs (negative vs. positive)	0.729	0.537	0.991	0.044

OS of 25.4 months (95% CI 13.5–37.3 months, $p = 0.005$). Similar results were found for the group of patients after neoadjuvant treatment; again, IDO serves as a significant prognostic marker ($p = 0.041$) with a median OS of 30.8 months (95% CI 12.0–49.6 months) in IDO-positive patients compared to a median OS of 22.4 months (95% CI 18.5–26.3 months) in IDO-negative patients.

4. Discussion

Here, we report the expression of the immune checkpoint protein IDO on tumor-associated inflammatory cells in a large and well-characterized cohort of 551 patients with EAC. We evaluated the level of heterogeneity and distribution of IDO-positive TILs within the tumor. IDO expression on TILs was a strong and statistically independent prognostic biomarker for an improved overall survival in EAC. IDO expression correlated significantly with low UICC stages (I/II) and nodal-negative status (pN-). Furthermore, we found a strong correlation with INF-γ expression and the number of CD3-positive T cells within the tumors. In multicolor immunohistochemistry, we demonstrated a predominant coexpression of IDO with CD4-positive T cells. No correlation of IDO expression on TILs with important molecular alteration markers like *TP53* mutational status and HER2/neu amplification was seen.

For the test cohort of 165 patients, we built a multispot TMA considering two different tumor localizations (surface and infiltration margins) proving low heterogeneity within the 4 spots of one tumor localization and a consistent expression pattern between surface and infiltration margins, respectively. Thus, the expression of IDO in randomly taken EAC samples by endoscopic tumor biopsy is most likely representing overall tumor IDO expression. Furthermore, the absence of significant heterogeneity was one reason to evaluate IDO expression on a single-spot TMA with 386 additional patients (551 in total) to confirm our results.

To the best of our knowledge, we are the first to evaluate IDO protein expression in EAC. Rosenberg et al. analyzed IDO mRNA expression using TCGA data of squamous cell cancer and adenocarcinomas of the esophagus correlating other checkpoint markers like PD-L1 and CTLA4 [18]. Overall, they found high IDO mRNA levels being associated with worse patient survival. However, the additionally performed IDO protein expression (IHC) in 93 patients with ESCC did not correlate with survival. For EAC, their results are solely based on mRNA data.

Within our cohort, IDO protein expression on TILs was an independent prognostic biomarker within all tumor stages indicating a tremendous survival benefit especially in pT1/2 tumor stages. Since we did not detect heterogeneity in the expression pattern, high levels of IDO expression on TILs in endoscopic biopsies could probably define a subgroup of patients with a favorable prognosis, possibly with no further benefit of radiochemotherapy in this group. This could be particularly interesting for patients in advanced tumor stages with an extremely reduced expectancy of life avoiding an aggressive therapy concept with reduced benefit. Neoadjuvant treatment did not influence the prognostic effect of IDO on overall survival in this cohort of EAC. In a mouse model, IDO was shown to increase after radiotherapy [30]. In non-small-cell lung cancer (NSCLC), there is evidence that radiotherapy indeed reduces IDO activity during therapy but increases posttherapeutically with worse prognosis in NSCLC [31]. However, in that study, IDO was not analyzed directly in the tumor tissue, but kynurenine serum levels were measured as an indicator of IDO activity.

Physiologically, IDO protein catalyzes the elimination of the essential amino acid tryptophan [32]. The resulting metabolites (l-kynurenine, l-hydroxykynurenine, 3-hydroxyanthranilic acid, quinolinic acid, and picolinic acid) were initially considered to protect the host from infections but have recently been recognized to provide regulatory effects on the inflammatory microenvironment [33]. Accumulating metabolites are supposed to cause immunosuppression by the activation of regulatory T cells, apoptosis of T effector cells, and inhibition of T cell proliferation [8, 33]. Immunosuppressive characteristics of IDO have therefore accounted for the establishment of IDO inhibition in clinical trials to encourage immune response against the tumor [34]. Even Opitz et al. describe the importance of tryptophan metabolism in targeted therapy concepts in a recently published study, although they conclude that IDO inhibition has failed as a sufficient therapy concept until today [35]. Nevertheless, several studies describing an adverse influence of IDO on patients' clinical outcome refer comprehensibly to these effects of an elevated IDO expression [10, 11, 36].

However, and in opposite to these findings, we clearly demonstrate not only a favorable impact of IDO expression on TILs on overall survival in a large cohort of 551 patients with EAC but also IDO to be an independent marker for prognosis. This is absolutely in common with former research, e.g., a recent study by Patil et al. [37] examined IDO expression in gastric adenocarcinoma and found comparable results concerning the amount of IDO-positive samples (58%; our test cohort: 57.9% and 59.6%, respectively) and a favorable prognostic impact of stromal IDO expression. An elevated IDO expression is further linked to an improved overall survival in breast cancer, renal cell carcinoma, and cervical cancer [38–40]. Therefore, alternative effects of IDO expression on cancer progression have been discussed. For instance, Soliman et al. assumed that a local decrease of tryptophan might lead to metabolic growth disadvantage for tumor cells [41]. Moreover, Riesenberg et al. [40] considered toxic metabolites of tryptophan elimination to damage tumor cells and found a significantly decreased proliferation in tumor cells exposed to IDO-positive microvessels. We further hypothesized that overexpression of regulatory proteins in the immune compartment of a tumor could be part of a generally elevated immune response of particularly immunogenic tumor biology. IDO is activated by INF-γ, and as we could point out by using RNA BaseScope technology, IDO-positive tumors were enriched with INF-γ-positive inflammatory cells in the tumor microenvironment. It is therefore thinkable that the favorable outcome of IDO-positive tumors is driven by the inflammatory microenvironment but not the immune checkpoint itself, which is rather representing a subsequent regulatory counterpart in that

reaction. Concordant to this, previous research on colorectal cancer found immune checkpoint expression of PD-1, PD-L1, CTLA-4, LAG-3, and IDO as a counterbalancing part of highly inflamed tumors (MSI unstable) [42].

Apart from IDO expression on inflammatory cells, we additionally found expression on tumor cells in 9.2%. IDO expression has been previously described in various cell types, including endothelial cells, mesenchymal stromal cells, fibroblasts, and various myeloid-derived antigen-presenting cells such as DCs and macrophages, as well as tumor cells [8]. Different results concerning the influence of IDO on tumor progression could therefore be dependent on the expressing cell type. In cervical cancer, Heeren et al. found differing serum levels of the immunosuppressive tryptophan metabolite l-kynurenine dependent on whether IDO is expressed on tumor cells (high levels of l-kynurenine) or immune cells (low levels of l-kynurenine) [39]. Riesenberg et al. further concluded that a selective enhancement of IDO expression in endothelial cells, but not in tumor cells, reduces tumor progression in renal cell carcinoma [40]. Therefore, our findings of a beneficial effect of IDO displayed exclusively on inflammatory cells underline the importance of an individual evaluation of IDO expression patterns for tumor cells versus stromal/inflammatory cells.

Still, open questions concerning the interaction of IDO and cancer growth need to be answered. For example, the significant decrease of IDO expression in advanced tumor stages remains cryptic. We assume that there is an interaction between immune checkpoint expression of the tumor microenvironment and invasive tumor growth. However, it is not clear whether overexpression of immune regulatory proteins negatively influences local tumor growth, or changes in tumor biology in advanced tumor stages might be responsible for a decreased level of checkpoints. Furthermore, the downstream metabolites of the tryptophan catabolism are known to influence IDO expression and activity and need to be considered in targeted therapy concepts [35].

Our study has strengths and limitations. We analyzed two independent and well-characterized cohorts of EAC. Furthermore, we identified an expression of IDO on inflammatory and tumor cells and discussed potential pro- and antitumoral effects. However, the study is retrospective and a selection bias cannot be excluded. We were not able to include patients who received neoadjuvant treatment and showed a complete tumor response or those with advanced tumors that were not eligible for surgical therapy. Beyond that, functional conclusions concerning biological mechanisms of IDO are not feasible on formalin-fixed material; therefore, future studies have to clarify the potential of IDO as a predictive biomarker in EAC.

5. Conclusions

In summary, our study describes the rate of IDO expression on TILs in EAC and demonstrates a strong and statistically independent positive prognostic effect in a very large group of EACs for the first time. IDO expression correlates significantly with low UICC stages (I/II) and negative lymph node status. However, prospective studies need to confirm our results. Since we find a favorable effect of IDO expression on overall survival in EAC, we assume that IDO interaction with tumor cells might be more complex than anticipated. Particularly, the IDO-expressing cell type as well as the metabolites of tryptophan catabolism might influence the effectiveness of future clinical trials investigating antibody-based IDO blockade in EAC.

Data Availability

All data of the study are available whenever requested.

Ethical Approval

All procedures performed in the current study involving human tumor specimens were in accordance with ethical standards of the local research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This retrospective study was performed according to the criteria of the ethics committee of the University Hospital of Cologne.

Disclosure

The funding exclusively provides financial support for student research assistance and had no influence on the conducted study itself.

Conflicts of Interest

All authors declare that they have no conflict of interest.

Authors' Contributions

Heike Loeser, Max Kraemer, Philipp Lohneis, and Alexander Quaas contributed equally to this work.

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PIK3CA and *KRAS* Amplification in Esophageal Adenocarcinoma and their Impact on the Inflammatory Tumor Microenvironment and Prognosis

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Abstract

Gene amplifications of *PIK3CA* or *KRAS* induce a downstream activation of the AKT-mTOR or RAF-ERK-pathways. Interactions of the active AKT pathway have been implicated in the inflammatory tumor microenvironment. Nothing is known about these interactions or prognostic power in esophageal adenocarcinoma (EAC). We retrospectively analyzed a large cohort of 685 EAC considering *KRAS* and *PIK3CA* gene amplification using fluorescence *in situ* hybridization (FISH) and immunohistochemistry. These results were correlated with clinical and molecular data as well as the inflammatory tumor microenvironment. Amplifications of *KRAS* were seen in 94 patients (17.1%), *PIK3CA* amplifications in 23 patients (5.0%). *KRAS* amplifications significantly correlated with nodal positive patients and poorer overall survival (OS) in the subgroup without neoadjuvant treatment ($p = 0.004$), coamplifications of *Her2* ($p = 0.027$), and *TP53* mutations ($p = 0.016$). *PIK3CA* amplifications significantly correlated with a high amount of tumor infiltrating T cells ($p = 0.003$) and showed a tendency to better OS ($p = 0.068$). A correlation with checkpoint makers (PD-L1, LAG3, VISTA, TIM3, IDO) could not be revealed. Our findings are the first to link the *KRAS* amplified genotype with lymphonodal positivity and poor prognosis and the *PIK3CA*-amplified genotype with a T cell–rich microenvironment in EAC. Future studies must show whether these two genotype subgroups can be therapeutically influenced. A dual inhibition of MEK and SHP2T could be effective in the subgroup of *KRAS* amplified EACs and an immune checkpoint blockade may prove to be particularly promising in the subgroup of *PIK3CA*-amplified EACs.

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Introduction

Esophageal carcinoma is the eighth most common diagnosed malignoma worldwide [1]. The incidence of esophageal adenocarcinomas (EACs) has increased rapidly mainly in the Western world over the past few decades [2–4]. Most of the adenocarcinomas arise from Barrett metaplasia due to chronic reflux disease followed by an accumulation of different mutations, copy-number variations, and chromothripsis causing genetic instability [5]. Despite improvements in perioperative treatments, the overall survival (OS) of patients with EACs remains poor showing a relative 5-year survival rate of about 20.2%. Among the most common mutations of the EACs are *TP53*, *CDKN2A*, and *ARID1a* [6].

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Interestingly, these and other mutations can already be detected in histologically inconspicuous Barrett's mucosa without dysplasia. The extent of mutations of a dysplastic Barrett mucosa is similar to that of EAC [7−10]. Another example for very early occurring mutations can be seen for *TP53* and *NOTCH1*, as such mutations are found in normal esophageal squamous cell epithelium in healthy volunteers. The probability of mutation increases with age and the extent of *NOTCH1*-mutations is greater than the expected rate of invasive esophageal squamous cell carcinoma [11].

There is growing evidence that copy-number alterations (CNAs) of the genome are the major pathophysiological differences between Barrett's mucosa and invasive adenocarcinoma of the esophagus [12,13]. The most common single loss-of-heterozygosity CNA seen in Barrett's mucosa is *CDK2NA* [14,15].

Copy-number gains (CNGs) are common in the EAC. According to the TCGA data, considering genomic data of approximately 185 EACs, there are putative CNGs among others in *Her2* (in up to 15%), *VEGFA* (14%), *EGFR* (14%), *MET* (4%), *c-MYC* (22%), *GATA6* (12%), *KRAS* (17%), and *PIK3CA* (18%) (compare http://cancergenome.nih.gov/) [16−18].

Activating mutations of *KRAS* and *PIK3CA* (or function-inhibiting genomic alterations of *PTEN*) increase the activity of important growth-promoting cancer pathways (RAS-RAF-ERK pathway or PIK-AKT-mTOR pathway). Various studies have shown that CNG/amplification of *KRAS* and *PIK3CA* lead to an activation of these pathways even without additional activating mutations in the genes themselves. According to the TCGA data and other publications, activating gene mutations and amplifications are exclusive (with rare exceptions) [19−21]. Recently, the therapeutic relevance of a *KRAS* amplification in wild-type (nonmutated) *KRAS* tumors of the upper gastrointestinal tract was highlighted and a therapeutic intervention with a combined inhibition of MEK and SHP2 was discussed [22].

Therapeutic interventions of the activated PIK-AKT pathway have been discussed as well and resistance to cisplatin-containing cytostatic therapy is described in ovarian cancer with amplification of PIK3CA mRNA [23].

There are no established findings on the prognostic significance of *KRAS* and *PIK3CA* amplification in primary resected or neoadjuvantly treated EACs and their correlation with the inflammatory tumor microenvironment [24].

In colon carcinoma and non−small-cell lung carcinoma, a relationship to mutations and CNA and specific reactions of the (inflammatory) tumor microenvironment was shown [25−29]. Interactions of the activated PIK-AKT pathway with the inflammatory tumor microenvironment have been shown in the past in other tumor entities like colon or ovarian carcinoma [30,31]. Activation of *AKT* leads to the recruitment of different inflammatory cells including CD8 positive T lymphocytes. This interaction is partly because of activation of the NF-kappaB pathway and activation of cyclooxygenase, leading to formation of prostaglandin E2, which enables its receptor to recruit certain T-cell subpopulations [32,33].

Particularly good response rates to immunotherapy can be found in gastric carcinoma or colon carcinoma in the group of microsatellite-instable tumors (MSIs), histologically typically associated with a strong inflammation in the tumor microenvironment, but this subtype is very rare in the EAC (1%) [29,34,35].

Nevertheless, we see a high variability in the extent of inflammation in the EACs of our tumor collection, but did not correlate molecular alterations of the carcinoma cells yet [36−39]. Thus we examined whether we find interactions between *PIK3CA* amplifications, leading to activation of the AKT pathway, and the recruitment of T lymphocytes into the tumor microenvironment of EAC. Furthermore, we evaluated the frequency of *KRAS* amplification in EAC. In addition, we analyzed the role of potential tumor escape mechanisms against T-cell recruitment by locally immunosuppressive checkpoint markers such as PD-L1, VISTA, LAG3, TIM3, and IDO. Therefore, we performed fluorescence in situ hybridization (FISH) and immunohistochemistry of 685 EACs, allowing us to accurately determine the extent of gene amplifications on a very large tumor cohort and correlated these results with clinical and additional molecular data and the composition of the inflammatory tumor microenvironment.

Material and Methods

Patients and Tumor Samples

We analyzed formalin-fixed and paraffin-embedded (FFPE) material of 685 patients with EAC in total that underwent primary surgical resection or resection after neoadjuvant therapy between 1999 and 2016 at the Department of General, Visceral and Cancer Surgery, University of Cologne, Germany. The standard surgical procedure consisted of a transthoracic en-bloc esophagectomy with two-field lymphadenectomy (abdominal and mediastinal lymph nodes), reconstruction was done by formation of a gastric tube with intrathoracic esophagogastrostomy (Ivor Lewis esophagectomy) [40]. The abdominal phase was predominantly performed as a laparoscopic procedure (hybrid Ivor Lewis esophagectomy). Technical details of this operation are described elsewhere [41−43]. Patients with advanced esophageal cancer (cT3, cNx, M0) received preoperative chemoradiation (5-FU, cisplatin, 40Gy as treated in the area prior the CROSS trial) or chemotherapy alone. The follow-up of all patients was performed according to a standardized protocol. During the first two years, clinical follow-up of patients was performed in the hospital every three months, followed by annual exams. These included clinical evaluation, abdominal ultrasound, chest X-ray, and additional diagnostic procedures as required. Follow-up data were available for all patients. Patient characteristics are given in Table 1. Depending on the effect of neoadjuvant chemo- or radiochemotherapy, there is a preponderance of minor responders in the tissue microarrays (TMAs), defined as histopathological residual tumor of ≥10% [44].

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

TMA Construction

For TMA, one tissue core from each tumor was punched out and transferred into a TMA-recipient block. TMA construction was performed as previously described [45,46]. In brief, tissue cylinders with a diameter of 1.2 mm each were punched from selected tumor tissue blocks using a self-constructed semiautomated precision instrument and embedded in empty recipient paraffin blocks.

Consecutive sections of the resulting TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ) for immunohistochemistry and FISH.

Analysis of Heterogeneity of Amplification

To clarify the important question of the heterogeneous distribution of amplified tumor clones, we further analyzed all amplified tumors

Table 1. Patients' Characteristics, *KRAS* ($n = 526$) and *PIK3CA* ($n = 461$) Amplification Status.

		KRAS				PIK3CA			
		Total	Negative	Amplified	*p* value	Total	Negative	Amplified	*p* value
Total		526	432	94		461	437	24	
		100%	82.1%	17.9%		100%	94.8%	5.5%	
Sex	Female	59	50	9		54	53	1	
		11.2%	84.7%	15.3%		11.7%	98.1%	1.9%	
	Male	467	382	85		407	384	23	
		88.8%	81.8%	18.2%	0.719	88.3%	94.3%	5.7%	0.339
Age group	<65 yrs	261	217	44		225	212	13	
		52.6%	83.1%	16.9%		48.8%	94.2%	5.8%	
	<65 yrs	235	188	47		212	201	11	
		47.4%	80.0%	20.0%	0.416	51.2%	94.8%	5.2%	0.836
Tumor stage	pT1	74	63	11		63	56	7	
		14.1%	85.1%	14.9%		13.7%	88.9%	11.1%	
	pT2	60	49	11		51	48	3	
		11.5%	81.7%	18.3%		11.1%	94.1%	5.9%	
	pT3	370	302	68		330	317	13	
		70.6%	81.6%	18.4%		71.7%	96.1%	3.9%	
	pT4	18	14	4		14	13	1	
		3.4%	77.8%	22.2%	0.880	3.0%	92.9%	7.1%	0.217
Lymph node metastasis	pN0	209	184	25		181	169	12	
		39.9%	88.0%	12.0%		39.4%	93.4%	6.6%	
	pN+	315	246	69		278	266	12	
		60.1%	78.1%	21.9%	0.004	60.6%	95.7%	4.3%	0.290
UICC stage	I	114	99	15		94	85	9	
		21.8%	86.8%	13.2%		20.6%	90.4%	9.6%	
	II	114	98	16		106	101	5	
		21.8%	86.0%	14.0%		23.2%	95.3%	4.7%	
	III	218	171	47		192	184	8	
		41.8%	78.4%	21.6%		42.0%	95.8%	4.2%	
	IV	76	60	16		65	63	2	
		14.6%	78.9%	21.1%	0.146	14.2%	96.9%	3.1%	0.198
Her2 status	Wild type	311	252	59		274	256	18	
		88.1%	81.0%	19.0%		87.8%	93.4%	6.6%	
	Amplified	42	40	2		38	36	2	
		11.9%	95.2%	4.8%	0.027	12.2%	94.7%	5.30%	1.00
TP53 status	Wild type	155	138	17		136	129	7	
		41.6%	89.0%	11.0%		41.6%	94.9%	5.10%	
	Mutation	218	173	45		191	179	12	
		58.4%	46.4%	12.1%	0.016	58.4%	54.7%	3.70%	0.812

on large tumor surfaces and constructed a heterogeneity tissue microarray (h-TMA) of 48 tumors. This h-TMA contains each three KRAS- and PIK3CA-amplified tumors, which were also analyzed on large surfaces, and additional 45 nonamplified tumors. Standard TMAs typically use one biopsy per tumor, whereas the h-TMA takes into account up to 12 biopsies per tumor. In tumors without lymphonodal metastases ($n = 25$) we punched out four biopsies from the tumor surface and four from the infiltration zone of the tumor periphery. In lymphonodal positive tumors ($n = 23$), four additional biopsies were taken from the lymph node biopsies.

Immunohistochemistry

The KRAS antibody (clone 9.13, Thermo Fisher, dilution 1:100) and PIK3CA antibody (clone 6D9, Thermo Fisher, dilution 1:1000) stainings were performed on TMA slides using the Ventana Benchmark stainer (Roche Diagnostics, Germany) according to the protocol of the manufacturers. Expression of KRAS and PIK3CA in the cytoplasm of carcinoma cells were assessed according to the following criteria: negative or weak staining in <5% of tumor cells (score 0); weak staining ≥5−20% of tumor cells (score 1); moderate to strong staining in ≥20% (score 2; compare Figure 3). The evaluation of immunohistochemical expression was assessed manually by two pathologists (A.Q. and A.E.). Discrepant results, which occurred in a small number of samples, were resolved by consensus review. Additional immunohistochemical markers were evaluated in this cohort of EAC, parts of it (VISTA, CD3) published [39], others (PD-L1, LAG3, TIM3, IDO) are currently under review. In brief, monoclonal antibodies were used (Ventana: PD-L1; Cell Signaling Technology: LAG3, TIM3, IDO) on the Ventana Benchmark stainer (PD-L1) or the Leica BOND-MAX stainer (Leica Biosystems, Germany) (LAG3, TIM3, and IDO). The expression in <1% lymphocytes were defined as negative and ≥1% was assessed as positive.

Fluorescence In Situ Hybridization

FISH for the evaluation of the *KRAS* gene amplification status was performed with the Zytolight SPEC KRAS/CEN12 Dual Color Probe (Zytovision, Germany) according to the manufacturers' protocol. For *PIK3CA* gene amplification analysis, the Zytolight SPEC PIK3CA/CEN3 Dual Probe Kit (Zytovision, Germany) was used according to the manufacturers' protocol. Sample processing was performed as described previously [47]. Tumor tissue was scanned for gene copy gains including chromosomal cluster amplifications hot spots using a 63× objective (DM5500 fluorescent microscope; Leica, Germany). In case the signals were homogeneously distributed, then random areas were used for counting the signals. Twenty tumor cells were evaluated by counting green *KRAS* or *PIK3CA* and orange centromere signals. The reading strategy for detecting amplifications

followed the recommendations *KRAS/CEN12* ratio >3.0 or *KRAS* extrachromosomal cluster amplifications signals [48]. For *PIK3CA* reading strategy followed the recommendations of previous studies *PIK3CA*/CEN3 ratio ≥2.0 or *PIK3CA* signals ≥5.0 proof an amplification [49,50].

Statistical Analysis

Clinical data were collected prospectively according to a standardized protocol. For statistical analysis, SPSS Statistics for Mac (Version 21, SPSS) was used. Interdependence between staining and clinical data was calculated using the chi-squared and Fisher's exact tests and displayed by cross tables. Survival curves were plotted using the Kaplan−Meier method and analyzed using the log-rank test.

Results

Clinicopathological and Patients' Characteristics

The entire cohort consisted of 685 patients with EAC were considered for analysis, in total there were 526 analyzable cases for *KRAS* (76.2%) and 461 cases for *PIK3CA* (67.3%) on the TMA. Reasons for noninformative cases included lack of tissue samples or absence of unequivocal cancer tissue in the TMA spot. Neoadjuvant treatment (either chemoradiation or chemotherapy) was administered in 403 patients (58.5%). The median follow-up for the entire cohort was 57.7 months with a calculated 5-year survival rate of 26.6% (compare Table 1 with patients' and tumor characteristics).

KRAS Amplification Status

KRAS amplifications were seen in 94 patients (17.1%). The amplification profile was not affected by administration of neoadjuvant treatment. Within the group of patients that underwent primary surgery, 41 patients showed *KRAS* amplification (18.1%) and 52 patients (17.7%) in the group of neoadjuvant treatment and resection ($p = 0.909$).

KRAS amplifications were not associated with sex ($p = 0.719$), patient's age ($p = 0.416$), and tumor stage ($p = 0.880$). Nodal positive patients (pN+) significantly correlated with a higher frequency of *KRAS* amplification ($p = 0.004$; Table 1). We found a correlation between *KRAS* amplification and *Her2* amplifications ($p = 0.027$) and *TP53* mutations ($p = 0.016$), respectively. A correlation between the inflammatory tumor microenvironment (CD3 positive T-cells, PD-L1, LAG3, VISTA, TIM3, IDO expression) could not be found.

PIK3CA Amplification Status

PIK3CA amplifications were seen in 23 patients (5.0%). Similar amplification rates were seen within the primary surgery group ($n = 10$; 4.9%) and surgery after neoadjuvant treatment ($n = 13$; 5.1%) ($p = 1.000$).

PIK3CA amplifications showed no correlation with sex ($p = 0.501$), patient's age ($p = 0.673$), tumor stage ($p = 0.155$), lymph node metastasis ($p = 0.669$), *Her2* amplifications ($p = 0.488$), or *TP53* mutations ($p = 0.132$). In tumors with a high amount of tumor infiltrating T cells (CD3 high), the frequency of *PIK3CA* amplifications was significantly higher (11.9%) compared with T-cell low tumors (3.0%, $p = 0.003$) (compare Figure 1). A correlation with any of the analyzed immune checkpoint makers (PD-L1, LAG3, VISTA, TIM3, IDO) could not be revealed.

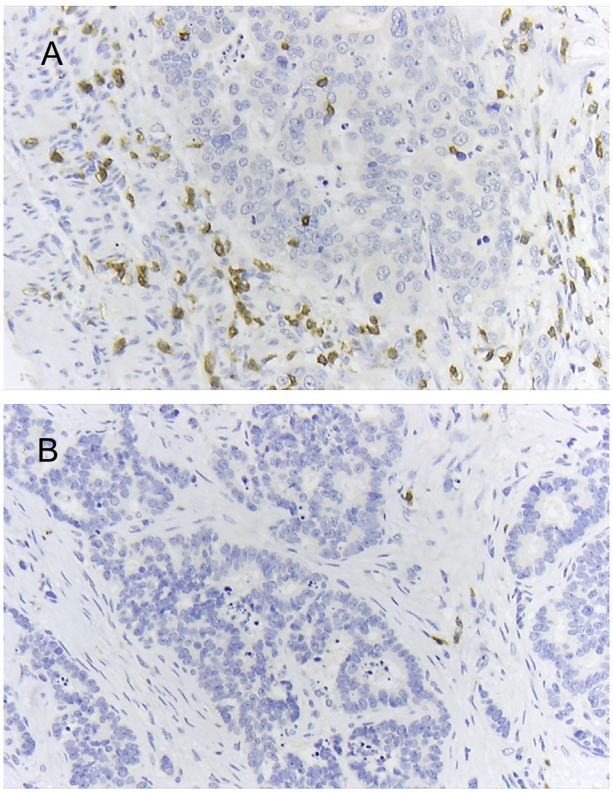

Figure 1. Esophageal adenocarcinoma showing (A) high amounts of tumor infiltrating CD3-positive T cells (brown cells) and (B) low content of CD3-positive T cells; magnification ×200.

KRAS and PIK3CA and Prognosis

Survival analysis on the entire patients' cohort did not show any difference in OS in dependence on the KRAS status (Figure 2). However, when analyzing the patient group with primary resection, there was a significant survival difference with a worse outcome for patients with *KRAS* amplifications. The median OS for *KRAS* nonamplified patients was 64.9 months (95% confidence interval (95%CI) 29.3−100.4 months) and for amplified patients 16.2 months (95%CI, 1.2−39.5 months, $p = 0.050$). For *PIK3CA*, no difference in OS was detectable for the entire patients' group ($p = 0.830$). Stratifying patients with and without neoadjuvant treatment did not reach a statistical significance. In patients without neoadjuvant treatment, median OS was 202.2 months (95% not achieved) compared with a median OS of 45.7 months (95% CI 26.2−65.2 months, $p = 0.068$).

A multivariate cox proportional hazard model revealed the presence of lymph node metastases as an independent prognostic marker ($p < 0.001$), but not *KRAS* or *PIK3CA* amplifications (Table 2).

Heterogeneity of Amplification

In *KRAS*, 15 amplified tumors showed homogeneous amplification across the tumor (both on the large tumor surface and on the h-TMA spots) and all nonamplified tumors were confirmed. In *PIK3CA*, three of the nine amplified tumors showed a heterogeneous distribution of the amplified tumor clones.

The heterogeneous distribution of *PIK3CA* also affects two cases with lymph node metastases that have both amplified and nonamplified tumor clones within the metastases.

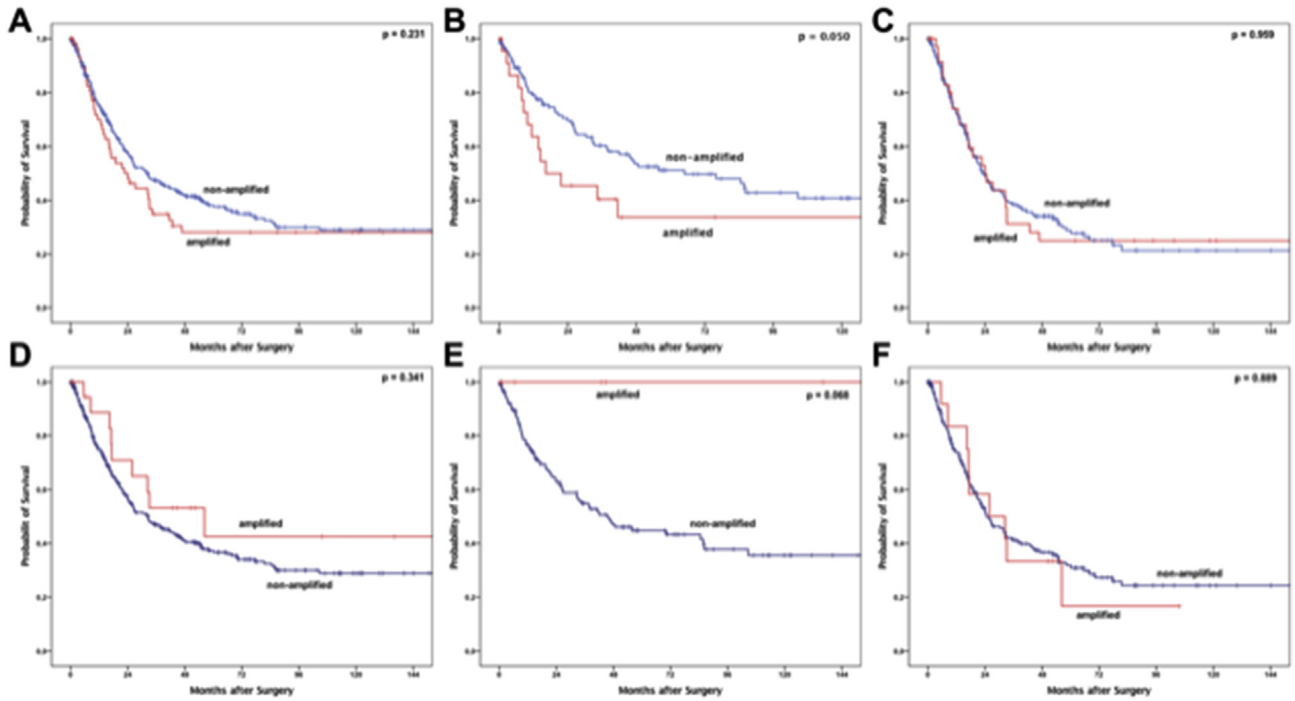

Figure 2. Overall survival using Kaplan–Meier analyses; (A) KRAS amplification for the entire patients cohort, (B) patients with primary surgery, and (C) patients with neoadjuvant treatment before operation; (D) PIK3CA amplification for the entire patients cohort, (E) patients with primary surgery, and (F) patients after neoadjuvant treatment.

Immunohistochemistry

All tumors that harbored *KRAS* and *PIK3CA* amplifications also expressed KRAS and PIK3CA protein visualized by immunohistochemistry (Figure 3).

Discussion

We found higher amplification rates for *KRAS* in our large tumor population than were reported in the TCGA cohort for adenocarcinoma of the esophagus [16,17], but confirm data from a recently published large study including tumors of the gastroesophageal junction [19].

To describe copy-number variations, differently suitable techniques can be used. FISH analysis allows us to precisely characterize the CNGs in tumor cells. FISH analysis is actually the current standard technique for evaluation of single-gene amplifications. We defined *KRAS* amplification according to internationally accepted criteria as a ratio of >3 and correlated our gene amplification results with protein expression of KRAS, which we determined by immunohistochemistry. We have found an excellent concordance between gene amplification and protein expression (Figure 3) and can confirm the results of a previous study describing the same concordance [19].

Table 2. Multivariate Cox-Regression Model.

	Hazard Ratio	95% Confidence Interval		
		Lower	Upper	*p* value
Sex	1.649	0.891	3.051	0.111
Age group (<65 vs. > 65 years)	1.249	0.925	1.686	0.146
Tumor stage (pT1/2 vs. pT3/4)	1.359	0.856	2.156	0.193
Lymph node metastasis (pN0 vs. pN+)	3.595	2.48	5.211	<0.001
KRAS (amplified vs. nonamplified)	0.83	0.567	1.217	0.341
PIK3CA (amplified vs. nonamplified)	0.924	0.482	1.771	0.811

We were able to show that *KRAS* amplified tumors represent a more aggressive subset of tumors in the primary resected, non-neoadjuvantly treated EACs mainly driven by the accumulation of lymph node metastases in this group.

This effect was no longer measurable in the neoadjuvant-treated group (combined radiochemotherapy according to the CROSS scheme or chemotherapy according to the FLOT scheme).

This is probably because of the already very unfavorable starting situation with higher clinical tumor stages and neoadjuvant pretreated pathohistological minor responders (= tumors which have an inadequate response rate with ≥10% vital tumor proportion) [44,51]. The minor responders are enriched in our collection of pretreated EACs, as in the prognostically more favorable complete responders no vital tumor is detectable in the surgical specimens, thus no tumor is present for further analyses.

In the cohort of EAC, we see no difference of *KRAS* amplification rates correlating the primary resected and the neoadjuvant subgroup, indicating no influence of neoadjuvant therapy concepts on the rate of *KRAS* amplifications.

We interpret the homogeneity of *KRAS* amplification as a signal of the biological relevance of this gene multiplication, probably underlining the high therapeutic effectiveness of pathway inhibition in these tumors.

In our collection, we observe an accumulation of *TP53* mutated and *KRAS*-amplified tumors as well as coamplifications of *Her2* and *KRAS*. Future studies will need to demonstrate whether personalized therapy with combinatorial inhibition of MEK and SHP2 is effective in the subset of *KRAS* amplified EACs as discussed most recently [19]. We do not see any impact on the inflammatory tumor microenvironment in *KRAS* amplified tumors.

PIK3CA amplification occurs less frequently than *KRAS* amplification in EACs. Primary resected patients with *PIK3CA* amplifications

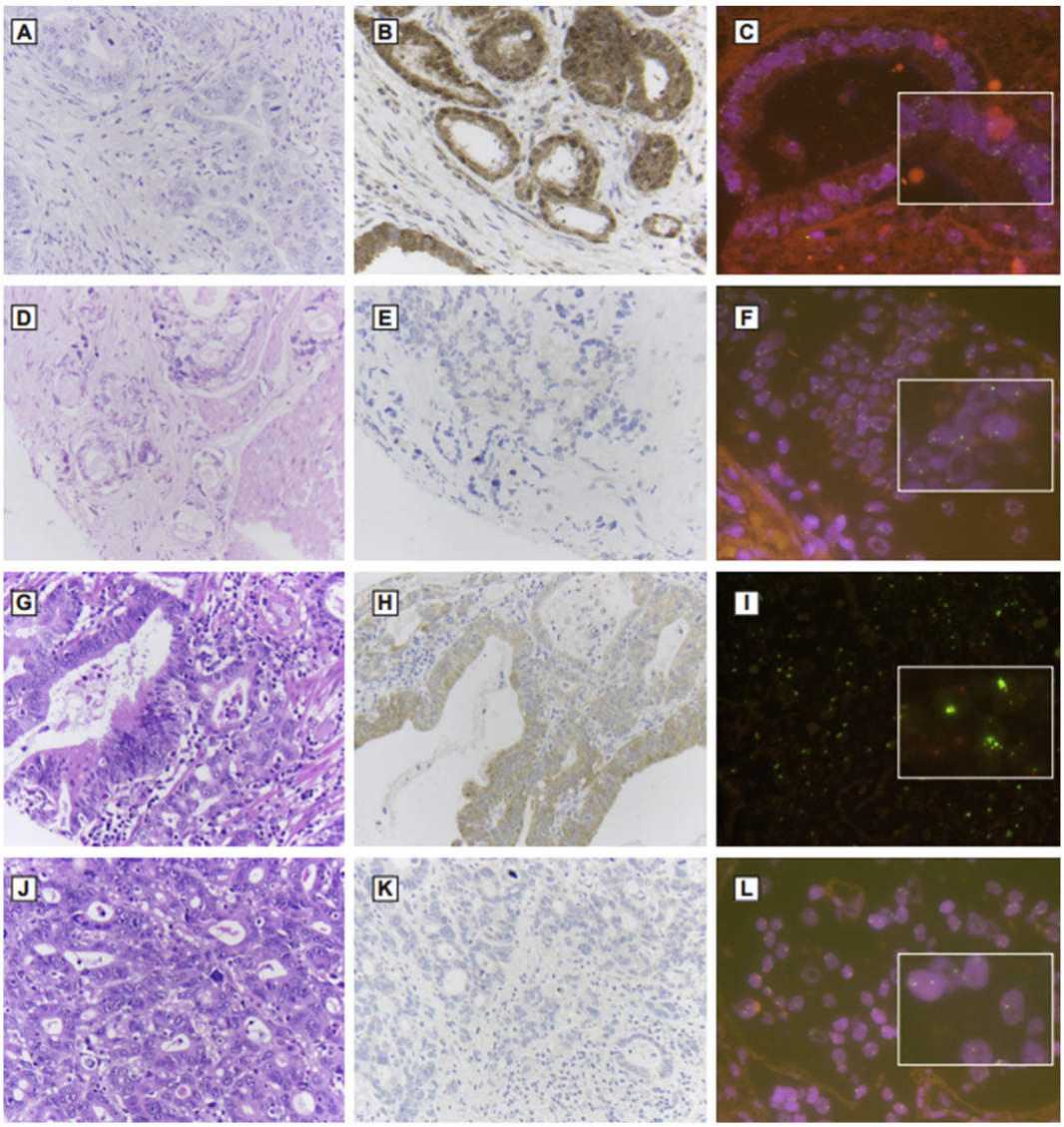

Figure 3. KRAS and PIK3CA gene amplification and protein expression, magnification x200. (A—C) PIK3CA positive: (A) esophageal adenocarcinoma (HE), (B) PIK3CA immunohistochemistry shows strong expression of the protein within the tumor, (C) *PIK3CA* amplification via FISH (red signals: centromer 3, green signals: *PIK3CA* gene). (D—E) PIK3CA negative: (D) Esophageal adenocarcinoma (HE), (E) PIK3CA immunohistochemistry shows no expression of the protein within the tumor, (F) *PIK3CA* nonamplified tumor via FISH with normal signal distribution pattern. (G—I) KRAS positive: (G) esophageal adenocarcinoma (HE), (H) KRAS immunohistochemistry shows strong expression of the protein within the tumor, (I) *KRAS* amplification via FISH (red signals: centromer 12, green signals: *KRAS* gene region with clusters). (J—L) KRAS negative: (J) Esophageal adenocarcinoma (HE), (K) KRAS immunohistochemistry shows no expression of the protein within the tumor, (L) *KRAS* nonamplified tumor via FISH with normal signal distribution pattern.

show a favorable prognosis; however, statistical significance is not achieved ($p = 0.068$). Conflicting results are found in the literature in esophageal squamous cell carcinoma. One work finds an unfavorable prognosis in *PIK3CA* amplifications, another study shows a favorable prognosis in activating *PIK3CA* mutations [52,53]. To our knowledge, nothing is known about the prognostic significance of *PIK3CA* amplification in EAC. A study on gastric carcinoma in Asian patients showed an unfavorable prognosis and a very high percentage of more than 60% *PIK3CA*-amplified gastric tumors [24]. Considering the inflammatory tumor environment, *PIK3CA*-amplified tumors have a significant accumulation of T lymphocytes. We observed in our tumor collection (data not published), as well as other studies, a favorable prognosis of T cell—rich inflammation in EAC [36—38]. The T-cell enrichment in the microenvironment may explain the tendency to the favorable prognosis of *PIK3CA*-amplified EACs.

This interesting relationship between gene amplification, tumor cell—associated cancer pathway activation, and effect on the inflammatory tumor microenvironment could be explained by the PIK-AKT pathway—mediated activation of the NF-kappaB pathway, as previously described on colitis-associated colon carcinoma or on ovarian carcinoma [30,31]. The activation of AKT is linked to an accumulation of inflammatory cells in the tumor microenvironment such as CD8 + T lymphocytes, whereas cyclooxygenase 2, which is also typically found on activation of this pathway, can regulate the synthesis of prostaglandin E_2 (PEG_2) leading to an accumulation of certain T cells via its PEG_2 receptor [32,33]. Because a T cell—rich

tumor microenvironment may be detrimental to the survival of the tumor, it is useful to establish escape mechanisms against T-cell inflammation. The upregulation of specific immunosuppressive checkpoint markers such as PD-L1, LAG3, IDO, TIM3, and VISTA may be helpful.

Therefore, we examined these checkpoint markers in our tumor population, but see no relation of an accumulation of these checkpoint markers and T cell−rich tumors with amplification of *PIK3CA*. Apparently, T cell−rich *PIK3CA*-amplified tumors are a subtype of EAC choosing other strategies of immune escape. Further studies need to clarify the interactions of copy-number variations and the tumor microenvironment.

Possible limitations of our work are the retrospective nature of the analyzes, the restriction to surgical specimens, and the accumulation of minor responders in our group of neoadjuvant-treated patients. Statements on the distribution of *KRAS*- or *PIK3CA*-amplified tumors in nonpretreated biopsy specimens and correlation to complete responders after neoadjuvance cannot be made.

Conclusions

In conclusion, this work on a very large collection of EAC shows that *KRAS* amplification is prognostically unfavorable in primary resected EACs, whereas the *PIK3CA*-amplified genotype with T cell−rich inflammatory tumor microenvironment shows a tendency to a better OS.

Future studies must show to what extent these two different tumor subgroups can be therapeutically influenced in the EAC. Thus, in the subgroup of *KRAS*-amplified EACs a dual inhibition of MEK and SHP2 and in the subgroup of *PIK3CA*-amplified EACs an immune checkpoint blockade may prove to be particularly promising.

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Declarations of Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.10.013.

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RESEARCH ARTICLE Open Access

HER2/neu (ERBB2) expression and gene amplification correlates with better survival in esophageal adenocarcinoma

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Abstract

Background: HER2 (ERBB2 or HER2/neu) is a tyrosine-kinase increasing cell proliferation. Overexpression/amplification of HER2 is correlated with worse prognosis in solid malignancies. Consequently, HER2 targeting is established in breast and upper gastrointestinal tract cancer. There are conflicting data concerning the impact of HER2 overexpression on esophageal adenocarcinoma (EAC), as most studies do not differ between cancers of the esophagus/gastroesophageal junction and the stomach. The aim of this study was to analyze the expression/amplification of HER2 in EAC in correlation to clinicopathological data to verify its prognostic impact.

Methods: We analyzed 428 EAC patients that underwent transthoracic thoraco-abdominal esophagectomy between 1997 and 2014. We performed HER2 immunohistochemistry (IHC) according to the guidelines and fluorescence-in-situ-hybridization (FISH) for IHC score2+, using tissue micro arrays (TMA) with up to eight biopsies from the surface and infiltration area of a single tumor for evaluating HER2-heterogeneity and single-spot TMA. The HER2-status was correlated with clinicopathological data.

Results: HER2-positivity was found in up to 14.9% in our cohort (IHC score 3+ or IHC score 2+ with gene amplification) and demonstrated a significantly better overall survival (OS) in correlation to HER2-negative tumors (median OS 70.1 vs. 24.6 months, $p = 0.006$). HER2-overexpression was more frequently seen in lower tumor stages (pT1/pT2, $p = 0.038$), in the absence of lymphatic metastases (pN0/pN+, $p = 0.020$), and was significantly associated with better histological grading (G1/G2) ($p = 0.041$).

Conclusion: We demonstrated a positive prognostic impact of HER2 overexpression in a large cohort of EAC, contrary to other solid malignancies including gastric cancer and breast cancer, but consistent to the results of a large study on EAC from 2012.

Keywords: HER2/neu (ERBB2), Esophageal adenocarcinoma (EAC), Prognosis, Immunohistochemistry, Tissue microarray (TMA), Fluorescence-in-situ-hybridization (FISH)

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Background

Esophageal adenocarcinoma (EAC) is a fatal disease with high mortality and increasing incidences in the Western world [1–3]. Although therapeutic procedures and treatment concepts have evolved, resulting in a trimodality therapy including neoadjuvant chemoradiation followed by radical oncological surgery, prognosis still remains limited [4–6]. Significant efforts have been made in this field, resulting in the description of a large variety of different putative markers, but so far, only one of them has made its way into the guidelines for targeted therapy for patients with advanced gastroesophageal adenocarcinoma, namely the human epidermal growth factor receptor 2 (HER2) [7].

The HER2 (also known as ERBB2 or HER2/neu) is a member of the epidermal growth factor receptor (EGFR) family and encodes a 185-kDa transmembrane tyrosine kinase receptor [8, 9]. Physiologically, HER2 is expressed in several tissues such as the nervous system, epithelial cells, or the mammary gland, where it promotes cell proliferation, controls differentiation, or suppresses apoptosis [9–11]. In case of uncontrolled activation of its associated pathway, this might result in excessive cell growth, angiogenesis, and tumorigenesis [11, 12]. Until today, HER2-overexpression/gene amplification has been detected in multiple solid tumor entities including breast cancer, lung cancer, glioblastoma, head and neck cancer, pancreatic cancer, colorectal cancer, gastric cancer, or EAC [12]. Trastuzumab is a humanized monoclonal antibody selectively targeting against HER2 at its extracellular domain of the receptor, resulting in an antibody-mediated cellular cytotoxicity [13]. In patients with HER2-positive advanced gastric or esophagogastric junction adenocarcinoma, the use of trastuzumab in combination with chemotherapy is a standard therapy concept and has a positive prognostic effect compared to chemotherapeutic treatment alone [14]. However, current data considering the prognostic role of HER2 in EAC are still controversial [15–19]. According to the current literature, the rate of HER2 positivity in EAC varies, ranging from 15 to 29% [19–24].

In this context, the aim of the present study is to analyze the expression/gene amplification as well as the distribution of HER2 in our large EAC cohort in correlation to the corresponding clinicopathological data, with the aim to verify the incidence and prognostic impact in this specific tumor subgroup.

Methods

Patients and tumor samples

To assess the distribution and heterogeneity of HER2, we created a multi-spot tissue array (TMA) with 165 tumor cases, according to the suggestions of the international immunooncology working group for assessing tumor infiltrating lymphocytes (TILs) in solid tumors. Four tissue cores from each tumor were punched out (diameter 1.2 mm) from the tumor surface/−center and from the deep-infiltration margin, respectively, and transferred into a TMA recipient block. For more detailed information, see Simon et al. and Helbig et al. [25, 26]. We defined the infiltration margin as the tumor areal showing the widest spatial distance from the mucosa-surface. In a second step, we used a TMA with single tumor punches of a further collective of 428 tumor cases for validating our HER2 analyses from the multi-spot TMA. For this, 4-μm sections of the resulting TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, NJ) for standard HE-staining. This retrospective study was performed according to the criteria of the ethics committee of the University Hospital of Cologne.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on TMA slides. For HER2, the Federal Drug Association (FDA) approved ready-to-use antibody (Pathway anti-Her2/neu rabbit monoclonal primary antibody, clone 4B5, Ventana) on the automated Ventana/Roche slide stainer was used. Membranous expression of HER2 in carcinoma cells was assessed according to the criteria for biopsies [27]: negative or staining in < 5 cells (score 0); very weak staining in cell groups ≥5 (score 1+); weak to moderate complete/basolateral/lateral staining in cell groups ≥5 (score 2+); strong complete/basolateral/lateral staining in cell groups ≥5 (score 3+). Scores 0 and 1+ display negative HER2 status, and score 3+ indicates positive HER2 status. Score 2+ has to be analyzed further by fluorescence or chromogenic in situ hybridization (see Fig. 1). The evaluation of immunohistochemical expression was assessed manually by two pathologists (A.Q. and H.L.). Discrepant results, which occurred only in a small number of samples, were resolved by consensus review.

Fluorescence in-situ hybridization

Fluorescence in-situ hybridization (FISH) for the evaluation of the HER2 gene amplification status was performed with the Zytolight SPEC ERBB2/CEN 17 Dual Probe Kit (Zytomed, Systems GmbH, Germany) according to the manufacturers' protocol. Sample processing was performed as previously described [28]. Tumor tissue was scanned for amplification hot spots using a 63x objective (DM5500 fluorescent microscope; Leica). In case the signals were homogeneously distributed, then random areas were used for counting the signals. Twenty tumor cells were evaluated by counting green *HER2* and orange centromere 17 (*CEN17*) signals. The reading strategy

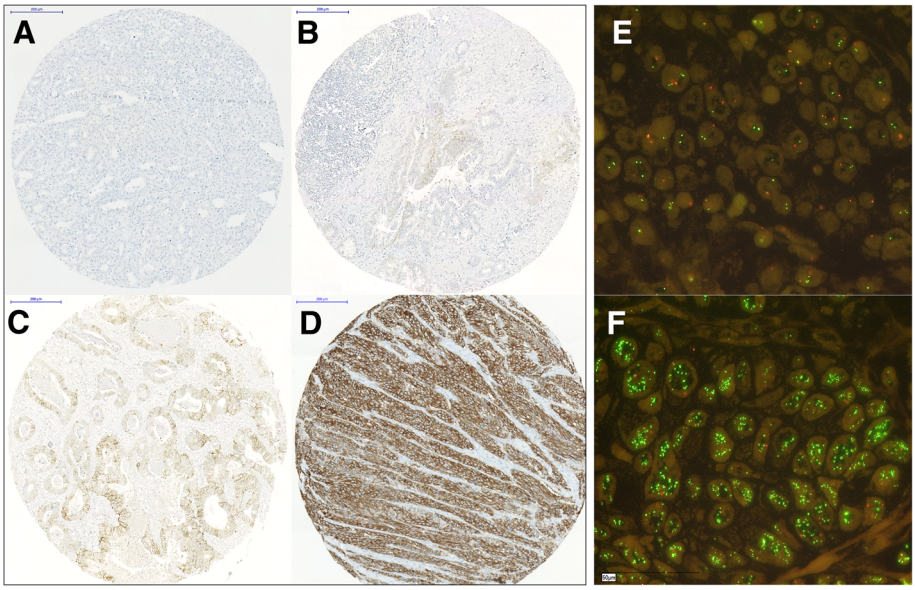

Fig. 1 Representative images of histopathological slides to illustrate the immunohistochemistrical scoring system **a**-**d**) as well as FISH-analyses (**e**, **f**): **a**) Negative or staining in < 5 cells (score 0); **b**) very weak staining in cell groups ≥5 (score 1+); **c**) weak to moderate complete/basolateral/lateral staining in cell groups ≥5 (score 2+); **d**) strong complete/basolateral/lateral staining in cell groups ≥5 (score 3+). Representative FISH-specimens **e**) without and **f**) with HER2 amplification

followed the recommendations *HER2/CEN17* ratio ≥ 2.0 or *HER2* signals ≥6.0 and negative ratio (< 2.0) [29].

Statistical analysis

Clinical data were collected prospectively according to a standardized protocol. For statistical analysis, SPSS Statistics for Mac (Version 21, SPSS) was used. Interdependence between staining and clinical data was calculated using the chi-squared and Fisher's exact tests and displayed by cross-tables. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. The heatmap for visualization was generated via Microsoft Excel for Mac; it is considered to visualize the heterogeneity within the tumor and is not a heatmap as commonly used for gene signatures (e.g., next-generation-sequencing (NGS)). Univariate and multivariate analyses were performed for prognostic factors of overall survival, using the Cox regression model. All tests were two-sided; P values < 0.05 were considered statistically significant.

Results

Patients' baseline characteristics

A total of 362 patients of 428 on the TMA with EAC that underwent surgical tumor resection were immunohistochemically interpretable on the single-spot and 161 patients on the multi-spot TMA. Reasons for non-informative cases (66 spots; 15.4% on the single-spot TMA, 5 spots 2.4% on the multi-spot TMA) included lack of tissue samples or absence of unequivocal cancer tissue in the TMA spot. Operative procedures were either thoracoabdominal en-bloc esophagectomy ($n = 274$, 64.0%) with intrathoracic anastomosis or transhiatal esophagectomy with transabdominal or cervical anastomosis ($n = 154$, 36.0%). Clinicopathological data is summarized in Table 1a. Median age at time of operation of the entire patient cohort was 64.9 years (range 33.6–84.5 years, average age 62.4 years, standard deviation +/− 10.7 years). The cohort was split into two groups according to age, above and below the median age, to conduct cross-table analysis revealing possible associations between patient age and clinical, respectively histochemical, data (see Table 1b). In total, 59.8% of the entire patient cohort received any kind of neoadjuvant treatment. Chemoradiation therapy was administered in 68.9% of those patients according to the CROSS protocol or modified CROSS, according to the individual patient's performance (reduction of either chemo- or radiation dosage). The remaining patients received chemotherapy upfront surgery according to the FLOT or the ECF protocol. On the single-spot TMA, 42 patients (9.8%) were female and 386 (90.2%) were male; a similar distribution was found on the multi-spot TMA (90.3% male, 9.7% female) The median age of the entire patient cohort was 65.2 years (range 33.6–85.6 years) at time of diagnosis. Neoadjuvant treatment (chemo- or radiochemotherapy) was administered to 253 patients (59.1%) before operation on the single-spot TMA and to 23 patients (13.9%) on the multi-spot-TMA.

Table 1 Clinicopathological data of the EAC-patients included in the study

				HER2 expression				*P* value
		Total		Negative		Positive		
a) Demographic and pathological results of the cohort								
SEX	female	38	10.5%	34	89.5%	4	10.5%	0.333
	male	324	89.5%	284	87.70%	40	12.3%	
Age group	< 65 yrs	195	53.8%	167	85.5%	28	14.5%	0.062
	> 65 yrs	167	46.1%	151	90.4%	16	9.6%	
Tumor stage	pT1	33	9.1%	26	80.0%	7	20.0%	0.038
	pT2	30	8.3%	28	93.9%	2	6.1%	
	pT3	287	79.2%	251	87.7%	35	12.3%	
	pT4	11	3.0%	11	100.0%	0	0.0%	
Lymph node metastasis	pN0	138	38.0%	113	82.5%	24	17.5%	0.02
	pN+	224	62.0%	204	91.1%	20	8.9%	
Grading	G1/2	152	61.8%	126	82.9%	26	17.1%	0.009
	G3/4	94	38.2%	89	94.7%	5	5.4%	
UICC	I	50	13.8%	39	78.8%	11	21.2%	0.039
	II	53	14.6%	47	89.1%	6	10.9%	
	III	169	46.7%	147	86.9%	22	13.1%	
	IV	89	24.6%	84	94.7%	5	5.3%	

b) Cross-table analysis of the patient cohort				
	Hazard ratio	95% confidence interval		*P* value
		Lower	Upper	
SEX (female vs. male)	1.718	0.977	3.023	0.06
Age group (< 65 vs. > 65 yrs)	1.194	0.914	1.559	0.193
pT (pT1/2 vs. pT3/4)	1.331	0.982	1.804	0.065
pN (pN0 vs. pN+)	0.937	0.77	1.139	0.513
UICC (Stage I/II vs. III/IV)	1.975	1.559	2.503	0
HER2 expression (neg. vs. pos.)	0.628	0.401	0.983	0.042

HER2 expression

The HER2 immunostaining was localized in the membranes of tumor cells. In total, HER2 positivity was detectable in 12.2% ($n = 44$) of interpretable EAC cases on the single-spot TMA. On the multi-spot TMA, HER2 expression was found in 24 patients (14.9%) on the surface area and in 18 patients (10.9%) at the infiltration margin.

The HER2 expression was correlated with lower pT-stages (pT1/2 vs. pT3/4, $p = 0.038$), low-grade stages (G1/2 vs. G3/4 $p = 0.041$), and the absence of lymph node metastasis (pN0 vs. pN+, $p = 0.020$). This reflects a correlation with early UICC stages, which was also significantly correlated ($p = 0.039$). In patients who underwent neoadjuvant treatment, HER2 expression was seen in a higher frequency than in patients without neoadjuvant treatment (21.2% vs. 9.2%, $p = 0.027$). Considering the heterogeneity of the HER2 expression, there was no significant difference between the surface and infiltration margins of the EAC specimens analyzed with a strong correlation between the expression status on the surface and infiltration margin (Fig. 2). A total of 133 patients were negative in both areas on the multi-spot TMA (84.2%), while 16 patients were double-positive (11.4%) ($p < 0.0001$).

HER2 as a prognostic biomarker

Patients with HER2 expression showed a superior overall survival (OS) compared to HER2-negative tumors. The median OS was 70.1 months (95% confidence interval (CI) 44.0–95.6 months) in HER2-positive tumors compared to a median OS of 24.6 months (95%CI 20.7–28.5 months, $p = 0.006$) in HER2-negative cases (Fig. 3). Patients that underwent neoadjuvant therapy showed lower HER2 expression than primarily resected patients (primary surgery 21% vs. neoadjuvant treated 9%). In the subgroup analysis, HER2 prognostic survival difference was only seen in the group of patients who

Fig. 2 Heatmap showing heterogeneity of HER2 expression between the luminal and the infiltration area of the primary tumor. Blue area represents absence of HER2 expression (IHC score 0); light red immunohistochemistry (IHC) score 2+, FISH confirmation negative; medium red IHC score 2+, FISH positive; dark red IHC score 3 +

underwent neoadjuvant treatment and not in the primary resected group.

Multivariate analysis

Multivariate cox-regression analysis revealed, beside the UICC stage, HER2 expression as independent prognostic with a hazard ratio of 0.628 (95% CI 0.401–0.983), displaying an improved OS in cases of HER2 expression ($p = 0.042$).

Discussion

A large diversity of different putative diagnostic or predictive biomarkers has been considered for EAC in the recent past. At least the status of HER2 is by now considered as a predictive marker for trastuzumab therapy

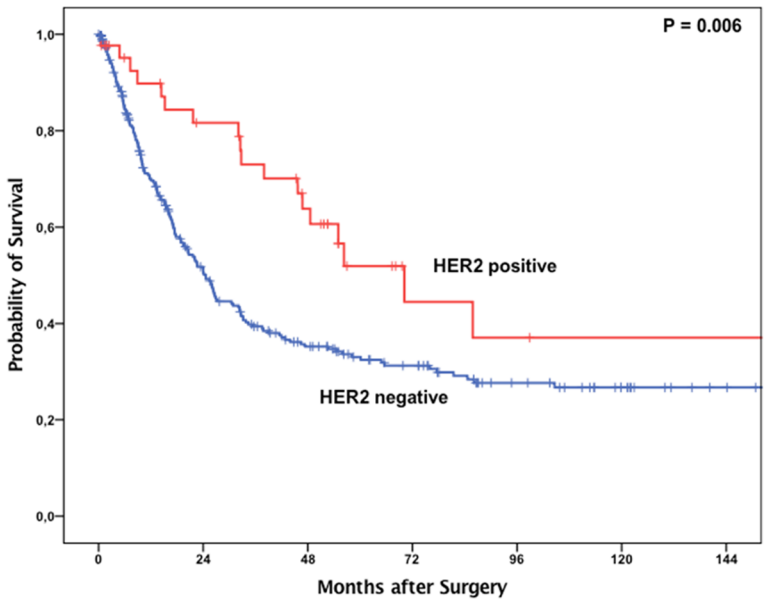

Fig. 3 Kaplan-Meier survival analysis (log-rank test) for the entire patient cohort on the single-spot TMA. Significant survival difference between patients with HER2 expression (median OS 70.1 months (95% confidence interval 44.0–95.6 months) compared to patients without HER2 expression (median OS 24.6 months (95% confidence interval 20.7–28.5 months), $p < 0.006$))

in advanced gastric and gastroesophageal cancer, since a prognostic therapeutic effect has been shown in the ToGA-study [14]. The HER2 physiologically controls cell differentiation or promotes cell growth via growth factor-induced signal transduction in several tissues. Consequently, dysregulated HER2 may cause tumorigenesis by suppressing apoptosis and by other effects [10–12]. A high expression of HER2 can be found in several solid tumor entities such as breast cancer, colorectal cancer, lung cancer, or pancreatic cancer [12]. The results considering HER2 in EAC within the upper gastrointestinal tumors are conflicting at first view, as most of the studies do not clearly distinguish between cancers of the esophagus/gastroesophageal junction and the stomach [24, 30–33]. In gastric cancer, HER2 overexpression is associated with poor survival and worse prognosis [12].

We found HER2 positivity in 14.9% (multi-spot TMA) and 12.2% (single-spot TMA) in our tumor cohort. This corresponds with the data published in literature reporting HER2 amplification/overexpression in 11–29% of all EAC-patients considered per analysis and identified via immunohistochemistry (IHC) and fluorescence-in-situ-hybridization (FISH), as done in the current study [18–24]. Both methods work well together, since a concordance of 93.5% between IHC and FISH has been described previously [34]. The variance of HER2 frequency is in mainly explained by different technical issues and divergent evaluation criteria as well as the low number of cases. We found comparable rates of HER2 overexpression/amplification in a large and well defined cohort of EAC [19]. Referring to the literature, HER2 immunoscoring differs not only between breast carcinoma and upper gastrointestinal adenocarcinoma, but there are different criteria for biopsies and surgical specimens of EAC/gastric adenocarcinoma (Table 2). Besides these conventional histopathological evaluation methods, the results of the current study correlate with the genetic data of Dulak et al., who performed NGS on 149 surgical specimens of patients with primary resected esophageal adenocarcinomas or cancers of the esophagogastric junction [35]. In their sequencing study, they identified HER2 mutation in 3% and HER2 amplification in 19% of the cases [35]. Similar results have been presented in 2017 by the Cancer Genome Atlas Research Network. Within their comprehensive molecular analysis of upper gastrointestinal adenocarcinomas, including 77 EAC tumors from patients who underwent primary resection, the authors demonstrated that HER2 amplifications took place in 19 cases (24.68%), while HER2 mutations occurred in three patients (3.9%). In an additional six cases of EAC (7.79%), multiple alterations of HER2 were detectable [36]. But not only in tissue specimens, but also in circulating tumor DNA, genomic

Table 2 Differences between HER2 evaluation in tumors of the upper gastrointestinal tract and breast cancer

	Tumors of the upper gastrointestinal tract	Breast cancer
Threshold	≥ 5 positive tumor cells of biopsies; ≥ 10% surgical specimen	≥ 10% positive tumor cells
Expression pattern	(baso)-lateral	circular

alterations of HER2 were found. Therefore, Kato et al. have analyzed 55 patients with advanced gastroesophageal adenocarcinomas via NGS, considering single nucleotide variants, copy number amplifications, fusions, and indels in selected genes [37]. Most of those patients did not qualify for surgical resection ($n = 46$), while 42 patients (67%) showed alternated circulating tumor DNA. Furthermore, the authors described HER2 alterations in eight cases (14.5%) of their study cohort.

In gastric and esophageal adenocarcinoma, HER2 shows a heterogeneous intratumoral distribution pattern; thus, some authors recommend a minimum of five biopsies to predict a precise HER2 status [34, 38, 39]. A recent study of HER2 expression in gastric adenocarcinoma and EAC revealed a heterogeneous expression in 27% of 15 paired biopsy and resection specimen, although only two of them showed a different overall HER2-status [40]. We therefore built our multi-spot TMA with up to eight tumor biopsies from the surface and infiltration area with comparable amounts of cancer cells according to endoscopically obtained biopsies to gain a reliable representation of the tumor heterogeneity, even exceeding the recommendation of five biopsies. In our cohort, we found no relevant heterogeneous expression of HER2, considering the spots of the multi-spot TMA neither within the same localization (infiltrative margin or surface) nor within the entire tumor (Fig. 1).

In the literature, most studies proclaimed a negative impact of HER2 on the patients' prognosis, as first described in breast carcinoma and which dramatically changed the standard therapy for numerous patients with the implementation of trastuzumab in the clinical routine [41, 42]. Many of the studies on gastrointestinal malignancies did not differ between different adenocarcinoma entities of the upper gastrointestinal tract, thus focusing on the prognostic impact of HER2 in EAC alone reveals ambivalent results [30–33]. Moreover, the therapeutic effects of a HER2 blockade in gastrointestinal tumors were rather disappointing compared to the strong benefits in breast cancer. In 2011, Langer et al. have demonstrated a significant negative prognostic effect of HER2 in 142 EAC for disease-free survival as well as overall survival and an association with poor tumor differentiation [20]. In a meta-analysis of 14 studies with either EAC or esophageal squamous cell carcinoma, the authors found a significantly poorer survival rate of HER2-positive EAC patients in studies with over 100 patients [22]. Consequently, the authors postulated HER2 to be a negative prognostic indicator in this context. A recently published analysis by Kato et al. drew the same conclusion in their NGS study of a total of 55 patients with mostly irresectable gastroesophageal adenocarcinomas [37]. Multivariate analysis in those irresectable patients revealed that detectable HER2 mutations within circulating tumor DNA were significantly associated with a poor overall survival compared to patients with the HER2 wild-type ($p = 0.003$) [37]. However, as mentioned before, maybe due to the small numbers of patients, the authors among Kato included different tumor entities within their cohort: On a closer look, only 11 patients had actual EAC tumors, while the other cases showed either gastric or gastroesophageal cancers [37]. Therefore, the transferability of these results to EAC in general seems to be limited in our opinion. In a published genomic characterization, the Cancer Genome Atlas Research Network identified no prognostic difference ($p = 0.781$) between those patients with HER2 amplification/alteration in upper gastrointestinal adenocarcinomas compared to those without (median overall survival: 31.28 vs. 28.75 months) [36]. Focusing on actual EAC tumors within the TCGA data set of this study, prognoses of 73 patients were available. Still, overall survival of this patient subgroup was not significantly altered in correlation to the HER2 mutation/amplification level ($p = 0.571$).

In contrast, another study, currently the largest one, considered an EAC cohort of 713 patients analyzed by Yoon et al., identifying HER2 positivity to be associated with better disease-specific survival and overall survival [19]. These results are fully in line with the results of our tumor cohort. From our point of view, HER2 expression in EAC indicates a biological favorable tumor behavior (early stage of disease, negative lymph node metastasis) and therefore hints to a certain tumor subgroup, associated with a better prognosis per se. This hypothesis is supported by the results of Yoon et al. [19].

In their work, Yoon et al. have demonstrated, via multivariate analysis, that the overall survival ($p = 0.0022$) as well as the disease-specific survival ($p = 0.0065$) among EAC patients who also had Barrett esophagus were independent of pathologic features such as tumor grade, depth of invasion, nodal status, and tumor location [19]. We obtained similar results within our study cohort: Multivariate cox-regression analysis revealed HER2 overexpression/amplification as an independent prognostic factor considering the overall survival.

Interestingly, the cohort of Yoon et al. included only patients who underwent primary surgery, while our study also considered patients with neoadjuvant therapy. This is concordant to the literature, where the differences of the HER2 status in pre-treatment biopsies compared to post-treatment surgical specimens are quantified with 5.9 and 6%, respectively [43, 44]. However, in a subgroup analysis, those patients who underwent neoadjuvant therapy showed lower HER2 positivity compared to the patients with primary resection, and the HER2 prognostic survival difference was only seen in the group of patients with neoadjuvant treatment. Thus, the data are also more consistent, since HER2 is expressed in early carcinomas,

nodal-negative, and G1/2 patients, and those are treated neoadjuvantly less frequently.

Although HER2 is a positive prognostic marker in EAC in our cohort, these tumors are still able to metastasize, and consequently, a selectively targeted therapy with trastuzumab should be initiated since it significantly improves the patients' prognosis compared to conventional chemotherapy alone [14]. However, hypothetically, the positive prognostic impact does not only derive from the pharmacological blockade of HER2, but also by those effects of the HER2-positive tumor subgroup itself.

With regard to the aspect of survival differences between patients with and without neoadjuvant therapy, the present study design naturally shows certain limitations. Patients with complete histopathological regression cannot be considered on the TMA due to missing tumor tissue and are therefore not included in the survival analysis. In addition, patients in advanced tumor stages are treated neoadjuvantly; those patients have a worse overall survival in advance than patients in early tumor stages who were not treated neoadjuvantly.

The major strengths of the current analysis are its large cohort size of patients with well-characterized EAC, including long-term follow-up data. By using TMA specimens for analysis due to the HER2 homogeneity detected in our cohort, this study also models the analysis of biopsy specimens, in which similar amounts of patient-derived materials are available for further diagnostics.

Conclusions

In conclusion, this study indicates the positive biological effects of HER2 positivity in EAC, being associated with better prognosis, earlier tumor stages, and a lower rate of lymphatic metastasis, representing a hitherto insufficiently characterized subtype of EAC.

Abbreviations

CEN17: Centromere 17; EAC: Esophageal adenocarcinoma; FDA: Federal Drug Association; FISH: Fluorescence-in-situ-hybridization; GCGC: Gastrointestinal Cancer Group Cologne; HER2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; NGS: Next-generation sequencing; OS: Overall survival; TILs: Tumor infiltration lymphocytes; TMA: Tissue micro arrays

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Availability of data and materials

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

Authors' contributions

PSP, FG, AQ, and HL conceived and designed the study; PSP, FG, HA, MK, FB, SHC, and LS enrolled the patients and collected the clinical data, while AQ and HL performed the pathological analysis; FG carried out the statistical analyses; PSP, FG, TZ, RB, AHH, CJB, AQ, and HL contributed to the interpretation of data. All authors were involved in writing the paper, critically revised the manuscript for important intellectual content, and approved of the submitted version.

Ethics approval and consent to participate

All procedures performed in the current study involving human tumor specimens were in accordance with the ethical standards of the local research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This was performed according to the criteria of the ethics committee of the University Hospital of Cologne.
Informed consent was not obtained because this secondary analysis of single protein staining is also regularly performed during pathological diagnostics.

Consent for publication

Not applicable.

Competing interests

Dr. Zander reports grants from the NRW Government during the conduct of the study; personal fees from Roche, personal fees from Novartis, personal fees from Lilly, personal fees from MSD, personal fees from BMS, personal fees from AstraZeneca, personal fees from Sanofi, outside the submitted work.
All other authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Immune profile and immunosurveillance in treatment-naive and neoadjuvantly treated esophageal adenocarcinoma

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Abstract

The outcome in esophageal adenocarcinoma (EAC) is still poor with only 20% of patients in Western populations surviving for more than 5 years. Almost nothing is known about the precise composition of immune cells and their gene expression profiles in primary resected EACs and also nothing compared to neoadjuvant treated EACs. This study analyzes and compares immune profiles of primary resected and neoadjuvant treated esophageal adenocarcinoma and unravels possible targets for immunotherapy. We analyzed 47 EAC in total considering a set of 30 primary treatment-naive EACs and 17 neoadjuvant pretreated (12 × CROSS, 5 × FLOT) using the Nanostring's panel-based gene expression platform including 770 genes being important in malignant tumors and their immune micromileu. Most of the significantly altered genes are involved in the regulation of immune responses, T-and B cell functions as well as antigen processing. Chemokine-receptor axes like the CXCL9, -10,-11/CXCR3- are prominent in esophageal adenocarcinoma with a fold change of up to 9.5 promoting cancer cell proliferation and metastasis. ARG1, as a regulator of T-cell fate is sixfold down-regulated in untreated primary esophageal tumors. The influence of the currently used neoadjuvant treatment revealed a down-regulation of nearly all important checkpoint markers and inflammatory related genes in the local microenvironment. We found a higher expression of checkpoint markers like LAG3, TIM3, CTLA4 and CD276 in comparison to PD-L1/PD-1 supporting clinical trials analyzing the efficacy of a combination of different checkpoint inhibitors in EACs. We found an up-regulation of CD38 or LILRB1 as examples of additional immune escape mechanism.

Keywords Esophageal adenocarcinoma · Immune profile · RNA expression · Nanostring

Abbreviations

ARG1	Arginase 1
CTA	Cancer testis antigen
CCR3	C–C chemokine receptor type 3
CXCL9	Chemokine (C–X–C motif) ligand 9
CD	Cluster of differentiation
CROSS	CROSS-trial regimen with paclitaxel, carboplatin and 41.4 Gy/23 fractions
CSM	Consensus molecular subtype
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DNAse	Deoxyribonuclease
EBV	Epstein–Barr virus
EAC	Esophageal adenocarcinoma
FC	Fold change

Svenja Wagener-Ryczek and Max Schoemmel contributed equally to this work.

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FFPE	Formalin-fixed and paraffin embedded
FLOT	FLOT-trial regimen with 5FU, Folinic acid, Oxaliplatin, Docetaxel
H&E	Hematoxylin–eosin
HAVCR2	Hepatitis A virus cellular receptor 2
HER2	Human epidermal growth factor receptor 2
HLA	Human histocompatibility leukocyte Ag
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon-gamma
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
LAG3	Lymphocyte-activation gene 3
MHC	Major histocompatibility complex
mRNA	Micro ribonucleic acid
MSI	Microsatellite instability
mAb	Monoclonal Ab
NACT	Neoadjuvant chemotherapy
NSCLC	Non-small-cell lung carcinoma
OS	Overall survival
PD-1	Programmed death 1 receptor
PD-L1	Programmed death ligand 1
RCT	Radio chemotherapy
RNA	Ribonucleid acid
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TGF	Transforming growth factor
TIL	Tumor infiltrating lymphocyte
TIS	Tumor inflammation signature
TMB	Tumor mutation burden
TNF	Tumor necrosis factor

Introduction

Esophageal adenocarcinoma (EAC) is associated with the sixth-highest cancer-related mortality and increasing incidences mainly in the Western World [1, 2]. Curative treatment consists mostly of a multimodal therapy of esophageal en-bloc resection and perioperative radio-chemotherapy, but compared to other cancer entities the outcome is still poor with only 20% of patients in Western populations surviving for more than 5 years [3–5].

There is a high need for new therapeutic approaches in treating this cancer [6].

The interaction of tumor cells and associated immune compartment is supposed to play an important role in cancer progression. Mechanisms of immunosuppression within the tumor and its microenvironment are incompletely understood, although neoantigen loss and negative regulation by immune checkpoints are presumed to lead to dysfunction of specialized T-cells [7, 8].

Immune checkpoint inhibitors (e.g. Pembrolizimab, Nivolumab) enhancing antitumor T-Cell activity through the inhibition of immune checkpoints, like the programmed death-1 (PD-1) receptor [9] and improved survival in some solid tumors like malignant melanoma and non-small cell lung carcinoma [10–13]. First line and second line treatment of metastatic esophageal cancer with checkpoint inhibitors considering the PD1/PDL1 axis are currently tested in a Phase III evaluation with pembrolizumab (KEYNOTE-062, KEYNOTE-061) [14] as well as nivolumab (CheckMate-577) in the adjuvant setting with various other approaches in all lines of therapy [15].

Almost nothing is known about the precise composition of immune cells and their gene expression profiles in primary resected EACs and also nothing compared to neoadjuvant treated EACs.

Due to the fact that most EACs are neoadjuvantly treated, the question arises as to what effects neoadjuvant treatment has on the local immune micromileu in carcinoma?

Accordingly one aim of our study was to analyze and compare the immune profile of primary resected as well as neoadjuvant treated esophageal adenocarcinoma and to unravel possible targets for immunotherapy as for example cancer testis antigens (CTA) that have been shown to exhibit characteristics important for tumorigenesis. Targeting such antigens may control cancer progression [16]. Additionally we compared the primary resected EACs in their regulation of genes known to be associated with response to PD-1/PD-L1 inhibitors. This so-called hot inflammation profile consists of 18 genes associated with a T cell-inflamed and IFN-γ-related response to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance [17].

To address this question, we used the NanoString technology. Nanostring's panel-based gene expression platform, in particular, considers 770 genes that have been described as important in malignant tumors and their immune micromileu.

Materials and methods

Clinical characteristics of study cohort

We analyzed formalin-fixed and paraffin embedded material of 47 patients with esophageal adenocarcinomas (EAC). More than 80% ($n=40$) had locally advanced stages of EAC (T2 or more) and were predominantely men (89%) between 45 and 85 years old at the date of surgery.

Thirty patients (64%) received primary surgical resection (without neoadjuvant treatment) between 2014–2017 at the Department of General, Visceral and Cancer Surgery, University of Cologne, Germany. Standard surgical procedure was laparotomic or laparoscopic gastrolysis and right transthoracic en-bloc esophagectomy with intrathoracic

esophagogastrostomy including two-field lymphadenectomy of mediastinal and abdominal lymph nodes or transhiatal extended distal esophagectomy with transabdominal intrathoracic or cervical anastomosis as described previously described [18].

Seventeen patients (46%) had received neoadjuvant (radio)-chemotherapy. Four patients received chemotherapy alone according to the FLOT protocol (docetaxel, oxaliplatin, fluorouracil/leucovorin), one patient according to the ECX protocol (epirubicin, cisplatin, capecitabine) and 12 patients a combined radio-chemotherapy according to the CROSS protocol (paclitaxel, carboplatin and 41.4 Gy/23 fractions). We considered patients with at least 50% remaining tumor tissue after neoadjuvant treatment. High quality RNA was extracted from tumor of all 47 patients for NanoString Analysis. Accordingly, RNA from tumor-free tissue of 10 patients in the primary naive tumor cohort was extracted as healthy normal control.

Macrodissection and RNA isolation

All samples were routinely formalin-fixed and paraffin embedded (FFPE) according to local practice. Histological specimens were evaluated by board certified pathologists. 10 µm thick sections were cut from FFPE tissue block for RNA extraction. Six sections of 10 µm thickness were deparaffinized and the tumor areas were macrodissected from unstained slides using a marked hematoxylin–eosin (H&E) stained slide as a reference. For extraction the Maxwell RSC RNA FFPE Kit was used on the Maxwell RSC (Promega) according to manufacturer's instruction, including DNAse digestion.

Expression analysis

Differential expression of immune related genes on mRNA level was determined using the NanoString PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). Isolated RNA was hybridised to a set of 770 specific and fluorescently labelled gene probes for 18 h @ 65 °C. Afterwards hybridisation products were prepared for cartridge loading on an nCounter PrepStation. Digital Counting of fluorescent signals was conducted using the nCounter Digital Analyzer. Afterwards data analysis including statistics was carried out with the nsolver3.0 software and the advanced analysis 2.0 package. 40 housekeeping genes within the panel facilitated sample-to-sample normalization.

Results

Immune profile in primary treatment-naive esophageal adenocarcinomas

We first analyzed a set of 30 primary treatment-naive EACs and compared these gene expression results to matched normal esophageal mucosa (Table 1). Most of the significantly altered genes are involved in the regulation of immune responses, T-and B cell functions as well as antigen processing (Sup. Figure 1 and 2).

Factors that interfere with the antigen processing machinery

Within the whole study population of primary and NACT treated tumors (see Table 1 for patients´ characteristics), expression of MHC class I and II genes was upregulated in EAC compared to normal tissue (Fig. 1a). Nevertheless, we asked whether inhibition of macrophage phagocytosis by MHC class I could be altered in esophageal tumor specimen by an alternative way to interfere with antigen processing. We thereby identified LILRB1 to be significantly upregulated (fold change 2, $p = 0.0005$). LILRB1 plays a major role as a receptor in the detection and simultaneous inhibition of MHC class I triggered phagocytosis (Fig. 1b).

T-cell status

In the group of therapy-naive tumors, the following deviations from the normal sample should be emphasized:

CTLA-4 expression is significantly increased in primary resected, treatment naive EAC ($p = 0.01$) (Fig. 1c) as well as the costimulatory molecules CD80 and CD86 (Fig. 1c, d), which trigger higher interaction with CTLA-4. Further costimulatory and checkpoint molecules that are significantly overexpressed are CD70, which interacts with CD27 and TIM-3 (HAVCR2), which is also known as an important checkpoint molecule (Fig. 1c). Noteworthy also CD38 is overexpressed in primary esophageal tumors and represents a known mechanism of resistance to PD-1/PD-L1 blockade (Fig. 1b).

T-cells in treatment-naive- and post-radiochemotherapy (NACT) samples

T cell enrichment as measured by CD3 expression was found to be upregulated in treatment naive EACs by a factor of three in comparison to normal tissue (Fig. 1b). In contrast, post-NACT-treatmed samples presented a decrease in CD3 expression in comparison to treatment-naive samples, but present still a twofold normal tissue expression.

Table 1 Patients' characteristics

Neoadj.therapy	Naive		CROSS		FLOT		Total	
$n=47$ (Eurasian)	*n*	%	*n*	%	*n*	%	*n*	%
	30	63,8	12	25,5	5	10,6	47	100
Sex								
M	26	55,3	11	23,4	4	8,5	41	87,2
F	4	8,5	1	2,1	1	2,1	6	12,8
Age								
> 50	29	61,7	9	19,1	5	10,6	43	91,5
< 50	1	2,1	3	6,4	0	0	4	8,5
T-stad								
(y)T1/2	10	21,3	4	8,5	1	2,1	15	31,9
(y)T3/4	20	42,6	8	17	4	8,5	32	68,1
N stage								
pN0	16	53.3	5	41.6	1	20	22	46.9
pN1	12	40.0	5	41.6	3	60	20	42.6
pN2	2	7.0	2	16.8	1	20	5	10.5
Number of pos lymphnodes	3.4 (0–21)		2.2 (0–12)		6,4 (0–14)		3.4 (0–21)	
Number of resected lymphnodes	30.9 (18–51)		29 (18–51)		34 (12–52)		30.8 (12–52)	
Tumor length	2.2 (1.0–4.5)		2,1 (1.0–4,1)		1,9 (0.9–3.9)			
Smoking								
Yes	13	43.3	1	9,1	0	0	14	
No	13	43.3	7	54,4	5	100	25	
Former smoker	4	13.3	4	36,5	0	0	8	

Expression of the T-cell co-receptor CD8, which binds the MHC-I on antigen presenting cells, is increased threefold in tumor tissue regardless of therapy. CD8a expression varies between individual patient samples, nevertheless they all show a higher expression compared to normal tissue. CD8b receptor chain expression had a higher intra-group variability with some specimen showing expression values closely to normal expression. NACT was determined not to influence expression of CD8a and CD8b (expression of both CD8 chains alpha and beta was determined to remain stable under NACT). Within the top 50 genes, determined to be differentially expressed post NACT versus primary treatment-naive EAC, none of the above mentioned genes are present (Fig. 2 and Sup Fig. 3).

Factors that interfere with the function of activated T cells

Expression of different immune checkpoints (CTLA-4, CD-276, TIM-3, LAG-3, PD-1 and PDL-1).

In the following we evaluate differences in expression between therapy-naive tumors compared to the normal tissue and to neoadjuvant pretreated tumors (NACT): As already indicated, CTLA-4 expression is 3.5–4fold increased in esophageal tumor specimen compared to normal tissue (Fig. 1c). We observed a low variability of CTLA-4 expression between different patient samples, except one tumor with a very high and one sample with a very low expression of CTLA-4. The immune checkpoint receptor CTLA-4 plays a crucial role in the negative regulation of T-cell activation of cancer cells to evade immune response and maintain self-tolerance. The PD-L1 expression in all (primary untreated as well as NACT)—tumor specimens investigated is only slightly increased compared to normal tissue (Fig. 1c). NACT treatment per se does not seem to have an effect on PD-L1 status. PD-1 shows a 2–3 fold expression in comparison to normal healthy tissue (Fig. 1c). Intra-group variation was astonishingly low except few outliers, known that PD-1 expression is heterogeneous in other cancer entities. PD-1 expression status does not seem to be different in NACT treated samples.

Besides CTLA-4 and PD-1/PD-L1, there are further upcoming immune checkpoints gaining attention in preclinical trials for specific blocking antibodies (e.g. LAG3, TIM-3).

HAVCR2 (TIM-3) exhibits a threefold increased expression in esophageal tumor samples compared to normal control samples (Fig. 1c). Further, we evaluated whether TIM-3 expression is influenced by NACT and found no differential expression between both patient groups. LAG3 shows a fourfold higher expression in primary untreated

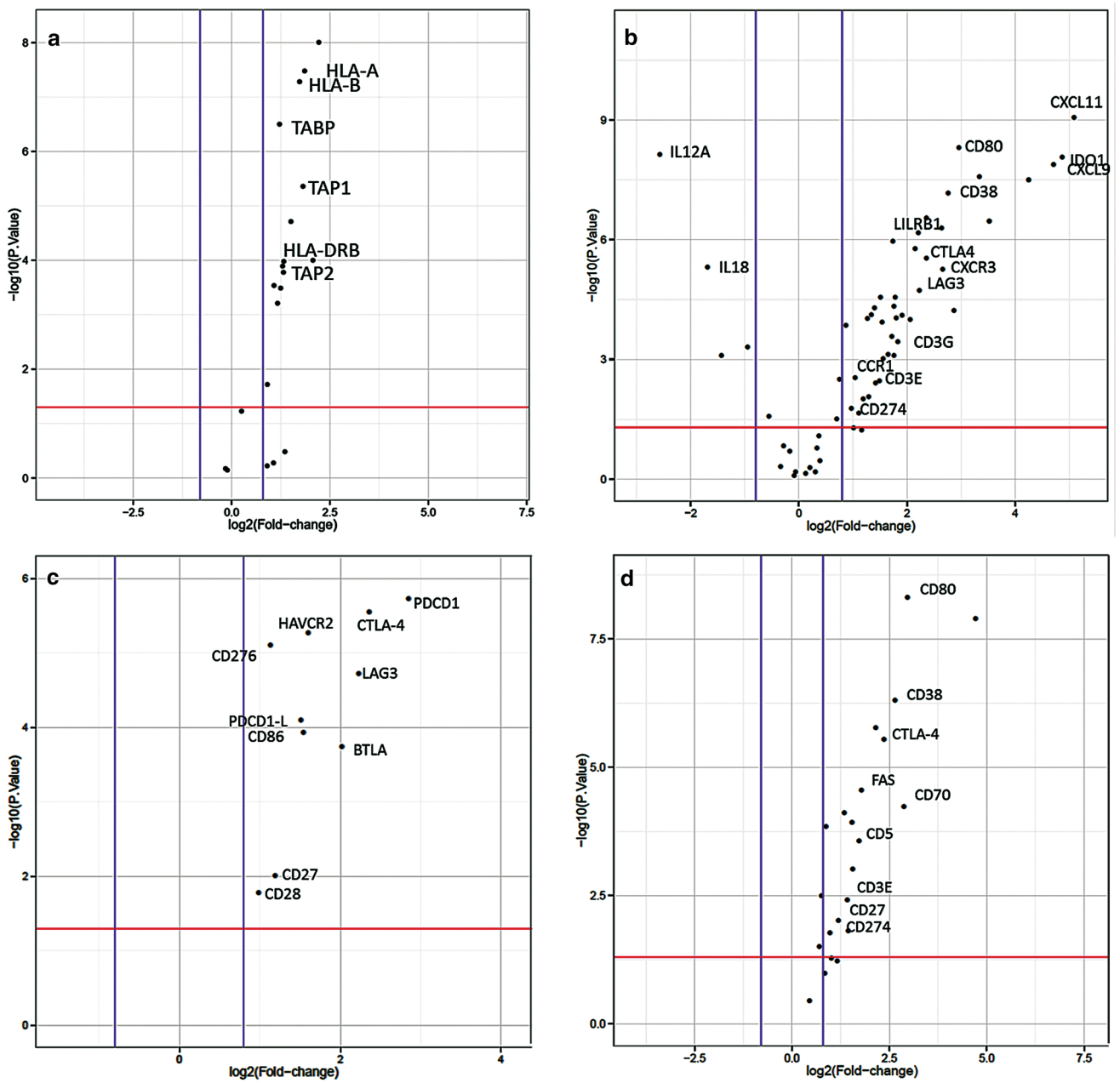

Fig. 1 Differential expression of immune-related genes. Upregulated expression of genes related to **a** antigen presentation. 'Volcano plot' of statistical significance against fold-change between primary EAC and normal tissue, demonstrating the significantly differentially expressed genes of MHC class I and II. **b** Expression of genes related to T-cell function. 'Volcano plot' of statistical significance against fold-change between primary EAC and normal tissue, demonstrating the significantly differentially expressed genes, CD3E, CD3G, LAG3, CTLA-4, LILRB1, CD38. **c** Expression of checkpoint genes. 'Volcano plot' of statistical significance against fold-change between primary EAC and normal tissue, demonstrating the significantly differentially expression of PDCD1, CTLA-4, HAVCR2, CD276, LAG3, PDCD1-L1, CD86, BTLA, CD27 and CD28. **d** Expression of genes related to B-cell function. 'Volcano plot' of statistical significance against fold-change between primary EAC and normal tissue, demonstrating the significantly differential expression of B-cell function related genes. Thresholds of significance *p*-value:1.3 (red line); log2FC: 0.8 (blue lines)

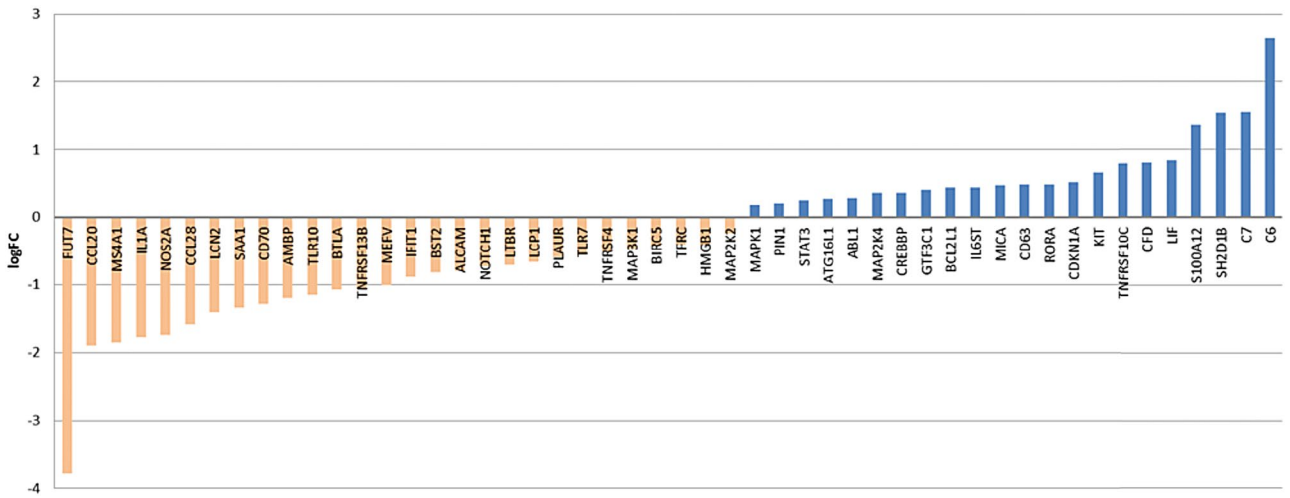

Fig. 2 Top 50 differentially expressed genes post NACT vs primary EAC. Waterfall plot for log2 fold-changes in mRNA gene expression levels in post NACTvs primary resected, treatment- naive EAC. Significantly downregulated genes are marked in orange and upregulated genes are marked in blue

esophageal tumor in comparison to healthy normal tissue (Fig. 1c). In contrast to HAVCR2, LAG3 expression is strongly reduced by the factor of two in the neoadjuvant treated group.

CD-276 (also known as B7-H3) showed a significantly increased expression in primary naive esophageal tumors as well. CD276 belongs to PD1-checkpoint family and elicits a similar inhibitory effect on T-cells as PD-1 does (Fig. 1c).

Secretion of immuno-modulating molecules

Noteworthy are in particular two markers (ARG1 and IDO1) that cooperate to establish an immunosuppressive tumor microenvironment. Both showed significant differences in expression to the normal tissue as well as to the neoadjuvant treated group:

ARG1, as a regulator of T-cell fate is sixfold downregulated in untreated primary esophageal tumors and fourfold downregulated in the NACT group in comparison to normal tissue.

IDO-1, which induces tolerance to self-antigens via inhibition of T cell activation, exhibits a sevenfold higher expression in primary untreated esophageal tumor in comparison to healthy normal tissue (Fig. 1b), whereas it has a fourfold higher expression in NACT treated patients. Thus representing a twofold downregulation of IDO-1 by NACT therapy. Nevertheless there is a high intra-group variability of IDO-1 expression in different patients with 2–3 fold higher expression values.

Factors that interfere with homing of activated T cells

Immune cells are regulated by many different chemokines. Therefore, we thought to investigate, which chemokine-receptor axes are prominent in esophageal adenocarcinoma samples. Interestingly we found the CXCL9, -10,-11/CXCR3 (fold change 9.5, 8, 14 and 2.5 with $p = 0.0005–0.0001$) axis to be significantly upregulated in the tumor tissue, promoting cancer cell proliferation and metastasis (autocrine axis) (Fig. 1b).

Expression of a Tumor inflammation signature that predicts response to immunotherapy

Primary untreated and neoadjuvant treated esophageal adenocarcinoma specimen were analyzed for their tumor inflammation signature, as developed by Merck and Nanostring to predict response to PD-1 blockade [19]. Afterwards samples were scored according to the sum of inflammation signature gene expression. Comparison of primary untreated to neoadjuvant treated esophageal adenocarcinoma specimen revealed HLA—and cancer testis antigens (CTA) expression to be comparable in both groups (Fig. 3a, b). Nevertheless, therapy-naive esophageal tumor specimen showed a numerical higher inflammation signatures as well as HLA expression (Fig. 3c). As described above, we could show a high CD38 expression within primary EAC. To unravel possible combinatorial treatments we determined whether CD38 expression correlates with the tumor inflammation signature (TIS) (Fig. 4). We analysed the probability and duration of survival for primary-resected and neoadjuvantly treated EAC patients as shown in the Kaplan-Meyer Curves

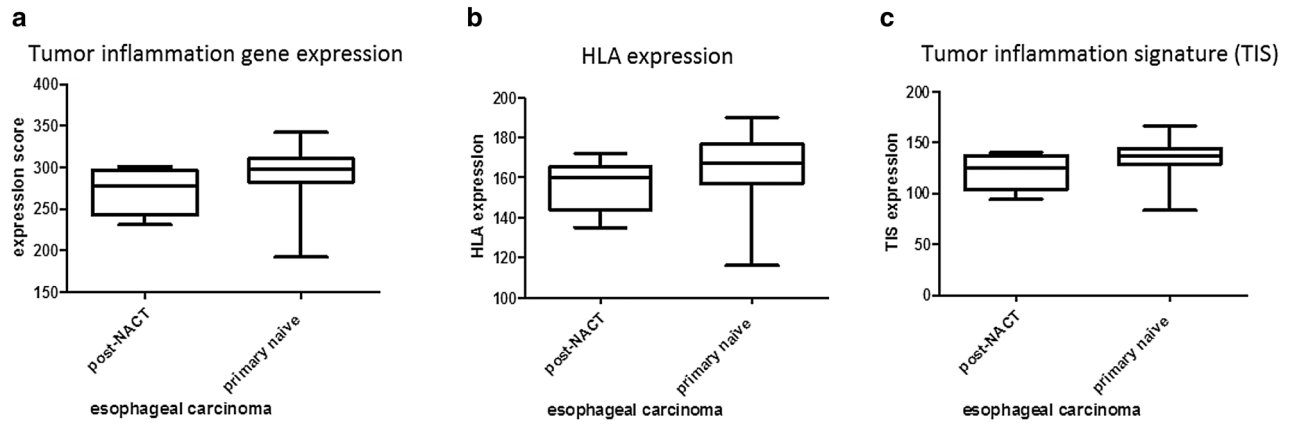

Fig. 3 Scored expression of HLA genes and tumor inflammation markers. Expression score of primary naive EAC and post NACT of combined HLA- and TIS expression (**a**), HLA expression (**b**) and tumor inflammation signature (TIS) as defined by Merck (**c**)

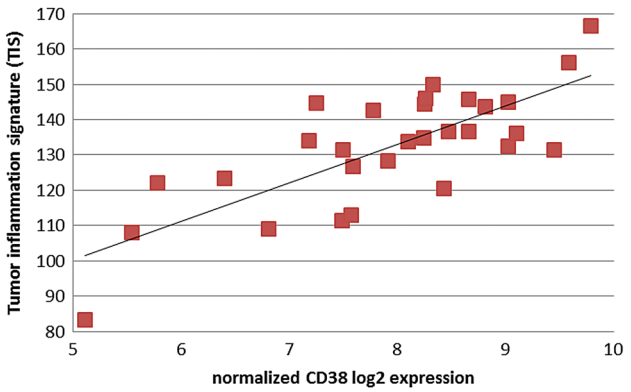

Fig. 4 Correlation of CD38 expression with the Tumor Inflammation Signature (TIS). Correlation coefficient $r=0.86$ indicates a strong correlation between CD38 expression and an inflammatory tumor phenotype as defined by the tumor inflammation signature (TIS)

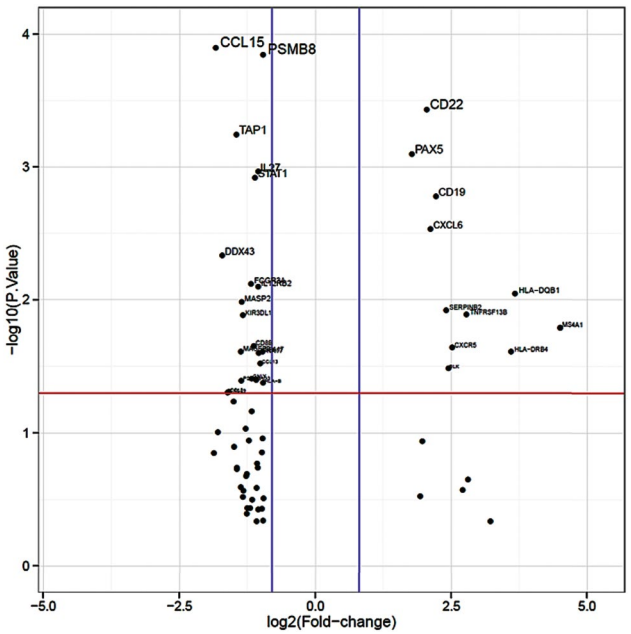

Fig. 5 Differentially expressed genes of FLOT-vs CROSS protocol treated patients. 'Volcano plot' of statistical significance against fold-change between post-NACT treated patients with FLOT vs CROSS protocol, demonstrating the significantly differentially expressed genes. Thresholds of significance *p*-value 1.3 (red line); log2FC: 0.8 (blue lines)

(Sup. Figure 4). Regarding the patients´ survival, there is a statistically significant difference between primary resected and the NACT-treated subgroup. A high inflammatory phenotype (primary EAC) correlates with a higher probability of survival, whereas NACT-treated tumors with a down-regulated immune response, show a reduce probability of survival (46 vs. 32 months).

Differences in immune related gene expression upon different perioperative therapy protocols (FLOT vs. CROSS)

We further asked, whether the type of neoadjuvant treatment protocol might have influenced the expression of central immune response related genes. Patient samples neoadjuvant pretreated with chemotherapy (FLOT) showed significantly upregulated gene expression of MHC class II molecules in comparison to neoadjuvant pretreated patients according to the CROSS protocol (radio-chemotherapy). Furthermore important factors of B cell activation (B cell receptor signaling) are significantly upregulated like the B cell receptor component CD79A, CD79B or its associated co-receptor genes CD19 and C21. In contrast we observed a

downregulation of MHC class I genes and the MHC I associated TAP transporter genes TAP1 and TAP2 (Fig. 5).

Discussion

Conventional oncological treatment regimens such as chemotherapy or radiotherapy are inadequate effective in EACs. Personalized therapy options are limited to HER2 blockage for a limited patient group showing a median advantage in progression-free survival of less than 3 months. Further therapy options are urgently needed.

Currently checkpoint-inhibitors like pembrolizumab and nivolumab which have proven to be effective, inter alia, in the treatment of malignant melanoma and NSCLCs are tested in different Phase III for esophageal cancer. First preliminary study results for pembrolizumab as a second-line treatment demonstrated an improved overall survival (OS) in patients with advanced or metastatic esophageal or esophagogastric junction carcinoma [20].

The structure of the studies available to date illustrates a significant problem. The studies mimic gastric adenocarcinomas with the adenocarcinomas of the esophagus (and subsume these as adenocarcinomas of the gastroesophageal junction) in the erroneous assumption that there are no relevant differences in tumor biology. As a matter of fact gastric adenocarcinomas reveal just for immunotherapy relevant subgroups such as microsatellite-instability (MSI) and Epstein–Barr-virus-related (EBV) subgroup, which are exceedingly rare or missing in adenocarcinomas of the esophagus [21, 22].

We have therefore focused on adenocarcinomas of the esophagus in this study.

The immune system interacts with esophageal adenocarcinomas in many ways and thereby substantially affects tumor progression and therapeutic response. Nearly nothing is known about these important interactions in EAC. Consequently the main focus of our study was to unravel the immune profile of EAC as defined by their T-cell activity, inflammation signature and immune escape mechanisms. Although most conventional therapies can elicit immune responses contributing to their efficacy, we could also show that radio-chemo therapy negatively alters the local immune status.

Other studies already identified molecular subtypes linked to the clinical outcome after immunotherapy. For example, different molecular subtypes have been identified in colorectal cancers which define potential strategies for immunotherapy [23]. Multiple characteristics are proposed to be responsible for a certain immune microenvironment as well as related mechanisms of immune escape. The consensus molecular subtype I (CSM I) for colorectal carcinoma is characterized by a high expression of PD-1, CTLA-4, IDO1 and other immune checkpoints. Moreover, its immune regulation is mainly driven by the chemokine CXCR3/CCR5 axis and cytotoxic effector mechanisms that are critical for activation and differentiation of T cells. CSM type IV in contrast is definied by an increased TGF-b signaling and upregulated CXCL-12, which drive inflammation and metastasis formation. Upon our findings, that primary untreated patients with EACs showed a high expression of major immune checkpoints as well as an upregulated CXCR3/CCR5 axis, it would be interesting to define prognostic phenotypes and thereby directing therapeutic strategies. In addition to this, we identified a subgroup of EAC patients with ultra-high expression of cancer testis antigens (CTAs), which displayed a significant upregulation of genes associated with tumor progression and metastasis formation. We therefore suspect, the score of CTAs to be a possible prognostic marker for clinical outcome in EAC as already identified for other tumor entities [16].

So far, neoadjuvant radiochemotherapy (RCT) is a well-established first-line treatment in patients with esophageal cancer. Nevertheless we here observed a significant decrease of T cell activity as measured by CD3 and CD8 expression after RCT. This finding implies that RCT impairs lymphocyte activity as well as components of the adaptive immune response, as targets of immunotherapy. Since the composition of the tumor microenvironment with immune cells and chemokines mainly drives efficacy of immunotherapy [24] and RCT profoundly suppresses the adaptive immune response, we propose that a combination of both could be restricted. Similar observations have been made in cervical and colorectal cancer patients [25, 26].

Restricting, however, we must state at this point that this explanation refers only to the local tumor micromileu. Memory cells in surrounding lymph nodes could trigger an effective neoantigen-driven tumor cell-destroying inflammatory response regardless of the local situation. Further it has to be kept in mind that the patient cohort of NACT-treated EAC includes only a small sample size. Nevertheless, these results can be an argument for clinical trials considering the use of checkpoint inhibitors first-line. We therefore propose to further validate the above described findings in future studies.

Controversely, we could not observe any upregulation of PD-1 expression upon chemotherapy as described previously [25, 26]. This might be due to the fact, that primary esophageal tumor samples show no differential expression of PD-1 and PD-L1 at all. This evidence further suggests that PD-1 blocking agents, which have shown to be promising in NSCLC and renal cancer as well as melanoma, might not be as effective in esophageal adenocarcinoma or at least just in a small subset of patients with EACs. Nevertheless, recent clinical trials reveal efficacy of checkpoint inhibitors also in PD-L1 low expressing patients. This phenomenon is

currently investigated [27] and noteworthy within our study, other checkpoint molecules like HAVCR2 (TIM-3), LAG-3 and CTLA-4 are dominantly expressed and therefore promising therapy markers/targets (see discussion below). Furthermore most EACs show a high mutational burden (TMB) which is correlated with good clinical response to checkpoint inhibition in NSCLC. Interestingly, a family member of PD-1, CD276 that even elicits similar inhibitory effects on T-cells is dominantly upregulated in primary EAC. Recently, CD276, also known as B7-H3, was identified to decrease levels of IFN-y, TNF alpha and inflammatory cytokines and thereby allowing immune escape [28].

Tumor escape from anti-tumor immunity is a critical event for tumor survival and progression [29]. Different mechanisms have been described and discussed extensively in the past [30, 31]. These include loss of antigenicity by modulation of the antigen presenting machinery. Downregulation of the antigen presenting MHC- class 1 has been found in various solid malignancies like melanoma, lung, breast and prostate cancers [32]. Primary EAC samples within our study cohort display increased MHC class I expression on mRNA level compared to normal tissue. In contrast IHC screening identified approximately 30% of EAC to have a loss of MHC marker expression on their tumor cell surface. Nevertheless we could identify other inhibitors of MHC class I-linked macrophage phagocytosis on mRNA expression level. Interestingly the major receptor in detection and simultaneous inhibition of MHC class I triggered phagocytosis, LILRB1, was significantly upregulated and could explain a possible tumor escape mechanism [33]. Further, tumors, which retain sufficient antigen presentation for immune recognition can still escape from elimination by downregulation of their immunogenicity, for example by the expression of immuno-inhibitory molecules (receptors and ligands) like PD-1/PD-L1, LAG3 and HAVCR2 (TIM-3) [30]). Also the microenvironment with infiltrating tumor lymphocytes (TILs) and T cell suppressing enzymes enhances immunoresistance. The ability of tumors to orchestrate this surrounding environment determines the cellular fate of TILs and allows evasion from immune elimination [34]. Enhancing efficacy of immunotherapy needs to consider immune escape mechanisms by immune profiling. Interestingly, within our cohort of primary naive esophageal carcinoma, distinct immune escape mechanisms are dominant, while others are not present. In detail, our cohort of primary EAC showed an upregulation of checkpoint inhibitors as most prominent mechanism of immune evasion with 7–4 fold increased expression of CTLA-4, HAVCR2 (TIM-3) and LAG3. Modulation of the tumor microenvironment as an enhancement of immunosuppression is prominent within primary naive EAC. We could identify high tumor inflammation signatures within nearly all patient samples compared to normal tissues. Approximately 50% of the samples elicit an extremely high score of inflammation markers. Furthermore, we could show a high CD38 expression within primary EAC, which was recently determined to be influenced by CD8 + T-cells within the tumor microenviroment and consequently correlates with the tumor inflammation signature [35]. This further strengthens current approaches to combine anti-CD38 with checkpoint inhibitor therapy [36]. Primary EAC, presenting a high inflammation signature in combination with dominant CD8 and CD38 expression might be promising targets for such a combinatorial treatment.

Recently, radiochemotherapy was thought to increase the presence of neoantigens as a result of its mutagenic character [37]. In general, a greater overall survival as well as higher efficiency of immunotherapy with checkpoint inhibitors are associated with higher neoantigen burden and CD8 + T cell infiltration [38]. Nevertheless, we identified that even the presence of cancer testis antigens is significantly decreased after radiochemotherapy in esophageal carcinoma. This is in concordance with another study conducted in ovarian cancer, where the authors found the predicted increase in neoantigens to be due to pre-existing mutational processes rather than from mutagenesis induced by chemotherapy [38].

The present study demonstrated some important new findings: (a) the influence of the currently used neoadjuvant treatment, (b) the unexpected higher expression of checkpoint markers like LAG3, TIM-3, CTLA4 and CD276 in comparison to PD-L1/PD-1 supporting clinical trials analyzing the efficacy of a combination of different checkpoint inhibitors in EACs, (c) the importance of immune escape mechanism like a high CD38 or LILRB1 expression in EACs.

TIM-3, also known as HAVCR2 could be an interesting and promising target for anticancer immunotherapy, since it is expressed on a variety of T-cells, DCs (dendritic cells), macrophages and monocytes and elicits a strong innate anti-tumor immune response. A variety of different studies have proven comparable effects of anti TIM-3 inhibition [39]. PD-1, TIM-3 and LAG-3 inhibitors are able to enhance the T-cell response to tumor antigens. Moreover a synergistic function of the above mentioned could enhance the response in combinatorial therapies [40, 41]. LAG-3 as a further promising immune-checkpoint has been investigated in various clinical trials and combinatorial treatment with anti-PD1 therapy showed high efficacy especially in PD1 resistant settings [40].

An increased expression of CD38 is correlated with a poor prognosis in chronic lymphocytic leukemia cells. Administration of the anti-CD38 mAb daratumumab has been shown to induce apoptosis and promotion of immune-initiated clearance [42]. A combinatorial screening of PD1/PD-L1 and CD38 could be of interest for diagnostics to predict response to PD-L1 blockade or even allow for

a combinatorial treatment with checkpoint inhibitors and CD38 blocking agents to improve patients' outcome.

Furthermore to further identify prognostic markers, a clinical follow up of patients with different immunoprofiles could be of high interest. Additionally to the relative low sample size within the cohort of NACT-treated EACs, it has to be kept in mind that this group is heterogeneous according to the type of treatment regimen. To strengthen the findings described within this study, a larger and homogeneous cohort of NACT-trated EAC patients could be tested in future research. Although heterogeneity of this sub-chohort, the herein described major influences of treatment to the immune profile are similar regardless of treatment regimen. There is a difference between FLOT and CROSS treated patients on gene expression as described in Fig. 5, but the major influence of NACT treatment (a down-regulation of nearly all important checkpoint markers and inflammatory related genes in the local microenvironment) is consistent between both subgroups.

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Author contribution SWR, MS and AQ designed the study, selected and reviewed cases and FFPE samples, conducted, analyzed and interpreted all experiments and wrote the manuscript. MK and HL helped with the review and processing of the FFPE sample cohort. CB, WS, TZ, FG and HA supported the project with patient material for the study cohort. SMB, RB, MT and HAS were involved in designing the project.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by the approval of the institution's human research review committee (Ethics Committee of the Medical Faculty of University of Cologne: registration no.13-091). Patients gave their written consent to usage of their tumor specimen.

Informed consent All patients gave written informed consent to the use of their tumors and their data for research and publication.

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7. Diskussion

7.1 Entwicklung einer Methode zur Identifizierung Kompartiment-spezifischer Biomarker

Das Single Cell Sequencing der RNA (scRNA-Seq) ist weithin die Methode der Wahl, um die Genexpression spezifischer Zelltypen zu analysieren [150, 151]. Um die Vorteile des scRNA-Seq gegenüber dem Bulk Sequencing in Hinblick auf die Bedeutung einzelner Tumorkompartimente zu bewahren und gleichzeitig größere Patientenkollektive berücksichtigen zu können, haben wir ein alternatives und kostengünstigeres Verfahren etabliert, welches Kompartiment-spezifische Transkriptomanalysen von EAC-Gewebe ermöglichen soll. Dabei wurde ein Ansatz gewählt, welcher nicht auf der RNA-Isolation aus Einzelzellen, sondern aus vorsortierten Zellpopulationen basiert. Im Prinzip wurden hierfür zunächst frisch gewonnene Proben aus EAC-Gewebe sowie korrespondierende Ösophagus-Mukosa mittels mechanischer und enzymatischer Dissoziation in Einzelzellsuspensionen überführt und bis zur weiteren Bearbeitung bei -80°C eingefroren. Im Anschluss daran wurden diese durch Fluoreszenzfarbstoff-markierte Antikörper gegen Epithel- (EpCAM), Fibroblasten- (PDGFRA, CD90, Anti-Fibroblast) und Immunzell-Epitope (CD45) gefärbt und FACS basiert in diese drei Zellpopulationen getrennt. Daraufhin wurde die RNA aus den Proben extrahiert, zur weiteren Lagerung bei -80°C eingefroren und anschließend Kompartiment-spezifisch sequenziert. Für genauere technische Details sei an dieser Stelle auf die Publikation „Cell type-specific transcriptomics of esophageal adenocarcinoma as a scalable alternative for single cell transcriptomics“ verwiesen [54].

7.1.1 Validierung des Verfahrens

Während unser Protokoll zukünftig die Identifizierung Kompartiment-spezifischer Biomarker durch einen Vergleich von Tumor- und Normalgewebe erlauben kann, nutzten wir die Sequenzierungsdaten der Proben, um das Verfahren zu validieren: Wir konnten eine erfolgreiche Separation in Immunzellen (durch CD45+), epitheliale Zellen (durch EpCAM+) und Fibroblasten (durch 2 von 3 aus PDGFRA, CD90, Anti-Fibroblast) anhand von vier nachfolgend aufgeführten Strategien zeigen. Dabei wurden die o.g. drei Zellpopulationen gegeneinander verglichen, unabhängig davon, ob es sich um Tumor- oder Normalgewebe handelte.

Eine zunächst durchgeführte *GeneOntology* Analyse zeigte passende biologische Funktionen für die einzelnen Zellpopulationen. Derartige Analysen sind ein allgemein anerkanntes Verfahren zur Strukturierung größerer Gendatensätze und können dabei helfen, zugrunde liegende Zelltypen zu identifizieren [152-154]. Hierfür erstellten wir eine Liste mit (gegenüber den anderen beiden Populationen) hochregulierten Genen für jede der drei Zellpopulationen. Diese „populations-spezifischen“ Gene ordnete die GO-Software unterschiedlichen Gruppen von biologischen Funktionen (sog. GO terms) zu, welche Kompartiment-spezifische Eigenschaften unserer Zielpopulationen zeigten (Beispiel: Hochregulierte Gene der CD45+ Zellpopulation wurden Gruppen wie „Immunantwort“ oder „Regulation von Immunsystem-Prozessen“ zugeordnet, so dass davon auszugehen ist, dass es sich bei den Zellen tatsächlich um Immunzellen handelt). Wir werteten die *GeneOntology* daher als wichtiges Argument einer gelungenen Separation.

In einer *Marker Gene Analysis* wurde das Vorkommen Kompartiment-spezifischer Gene in der Liste von hochregulierten Genen der drei Zellpopulationen überprüft. Wir konnten zeigen, dass Gene von Fibroblastenmarkern wie dem Fibroblast Activation Protein 1 (FAP1) und Alpha Smooth Muscle Actin (SMA) im PDGFRA/CD90/Anti-Fibroblast sortierten Zellkompartiment vermehrt exprimiert waren. CD45 positive Zellen zeigten eine vermehrte Genexpression Immunzell-typischer Oberflächenproteine (u.a. CD3, CD4, CD8 und CD19) und EpCAM positive Zellen hatten eine verstärkte Genexpression von Cadherin 1 (Genprodukt: E-Cadherin) und dem Epithel Membrane Antigen (EMA) als typische Epithelzellmarker. Das Vorkommen dieser Literatur-basiert entwickelten Markergene deutete ebenfalls auf eine suffiziente Trennung der Zellen in unseren drei Zielpopulationen hin.

Eine *Principle Component Analysis (PCA)* der Sequenzierungsdaten der drei sortierten Zellpopulationen zeigte weiterhin, dass eine Separation in drei unterschiedliche Zelltypen wahrscheinlich ist. Im Rahmen einer PCA werden multivariate Datensätze wie Genexpressiondaten durch bioinformatische Verfahren auf wenige Variablen reduziert, welche jedoch den Großteil der Varianz erklären [155]. Bei einer Reduktion auf zwei Hauptvariablen (principle components) ist eine zweidimensionale Darstellung von Clustern möglich, welche sich ähnelnde Datensätze repräsentieren. Wir konnten optisch eine eindeutige Trennung in drei unterschiedliche Genexpressionsmuster zeigen, welche mit den drei Zellpopulationen übereinstimmten.

Zuletzt wurde eine erfolgreiche Separation auch durch eine *Reference Component Analysis* belegt. Dabei wurden die RNA Sequenzierungsdaten unserer drei Zellpopulationen mit Referenz-Transkriptom-Daten von bekannten Zelllinien verglichen. Dieser 2017 ursprünglich für das Single Cell Sequencing entwickelte Algorithmus ist ein etabliertes Verfahren, um die Hauptzelltypen einer Gewebeprobe zu identifizieren [69]. Wir konnten zeigten, dass durch PDGFRA/CD90/Anti-Fibroblast sortierte Zellen eine hohe Korrelation mit mesenchymalen Zelllinien (z.B. mesenchymale Stammzellen, Fibroblasten) aufwiesen. CD45 positive Zellen korrelierten mit diversen Immunzelllinien (z.B. CD4/8 positive T-Lymphozyten, Makrophagen, Monozyten, Granulozyten) und EpCAM positive Zellen mit epithelialen Zelllinien (z.B. Kolonkarzinom-Zelllinien, bronchiales Epithel).

Eine erfolgreiche Separation in die drei Zielpopulationen mit anschließend suffizienter RNA-Extraktion stellt die Grundlage des von uns entwickelten Protokolls dar. Daher haben wir durch die vier dargestellten Strategien unsere Methode ausführlich validiert. Wir konnten zeigen, dass, wenngleich nicht auf Einzelzellebene, Zelltyp-spezifische Sequenzierungsdaten durch unser Verfahren alternativ zum Single Cell Sequencing generiert werden können.

7.1.2 Möglichkeiten und Verbesserungspotential

Durch die von uns entwickelte Methode präsentieren wir eine kostengünstige Alternative zum Single Cell Sequencing für eine Kompartiment-spezifische Transkriptom-Sequenzierung beim EAC. In unserem lokalen Setting berechneten wir, dass die Kosten eines Vergleichs der drei Zielpopulationen zwischen Tumor- und Normalgewebe durch unser Verfahren insgesamt 3,2-fach günstiger waren als eine derartige Analyse durch scRNA-Seq. Durch die damit entstehenden Kapazitäten können in zukünftigen Studien die Daten von größeren Patientenkollektiven analysiert werden, was zu einer stärkeren Validität der Ergebnisse beiträgt.

Darüber hinaus optimierten wir unser Protokoll auf eine Anwendbarkeit für besonders kleine Gewebemengen aus endoskopisch gewonnenen Biopsien. Somit können auch therapienaive Tumorbiopsien, welche im Rahmen der Diagnostik routinemäßig entnommen werden, mit normaler Ösophagus-Mukosa verglichen werden. Dies bietet die Möglichkeit, Kompartiment-spezifisch Alterationen zu identifizieren, welche nicht durch neoadjuvante Therapien beeinflusst sind und damit Aufschluss über Aspekte der initialen Tumorgenese des EACs geben. Zudem kann eine Korrelation mit klinischen

Daten von Patienten zur Entwicklung von Biomarkern in therapienaivem Gewebe beitragen. Diese könnten beispielsweise Aussagen zum erwarteten Therapieansprechen oder der Prognose erleichtern. Die Anwendung unseres Verfahrens an Material aus chirurgischen Resektaten (häufig neoadjuvant vortherapiert) wurde darüber hinaus ebenfalls gezeigt, so dass auch Vergleichsanalysen zwischen therapienaivem und neoadjuvant vortherapiertem Gewebe ermöglicht werden.

Trotz der beschriebenen Möglichkeiten und ökonomischen Vorteile besteht weiterhin Verbesserungspotential für das Protokoll. So konnte nur aus 31 von 45 (69%) angestrebten Zellpopulationen ausreichend Zell- und respektive RNA-Mengen für eine anschließende Sequenzierung generiert werden. Dabei war insbesondere der Verlust von Zellen durch das Einfrieren der Einzelzellsuspensionen von entscheidender Bedeutung. In Voruntersuchungen stellten wir eine Reduktion vitaler Zellen von bis zu 90% im Vergleich zwischen frischen und später aufgetauten Suspensionen fest (Daten in der Publikation nicht gezeigt). Interessanterweise stellte sich unser Verfahren dabei insbesondere für Tumorproben als robust heraus, wobei 20 der 21 (95%) angestrebten Zellpopulationen den gesamten Ablauf durchliefen. Dagegen schienen Proben aus Normalgewebe anfälliger für äußere Einflüsse zu sein, wobei nur 11 der 24 (45%) angestrebten Zellpopulationen am Ende ausreichend RNA-Material lieferten. Die Gründe hierfür bleiben unklar, wobei denkbar ist, dass Tumorzellen und ihr TME ausgeprägtere Überlebensstrategien entwickelt haben als Normalgewebe. Zur Verbesserung unseres Protokolls ist ein kontinuierlicher Arbeitsablauf ohne Einfrieren der Einzelzellsuspensionen daher zu empfehlen. In unserem Setting war dies aus logistischen Gründen leider nicht möglich.

7.1.3 Exemplarischer Vergleich Kompartiment-spezifischer Biomarker zwischen Tumor- und Normalgewebe

Das Ziel der Arbeit war in erster Linie die Entwicklung und Validierung der oben beschriebenen Methode. Daher haben wir, wie beschrieben, prospektiv Material aus einer kleinen Gruppe von Patienten gesammelt, um die erfolgreiche Zellseparation und RNA Sequenzierung zu demonstrieren. Die so gewonnenen Sequenzierungsdaten sind jedoch aufgrund der geringen Probenzahl nicht ausreichend, um generelle Aussagen zur Tumorbiologie beim EAC zu treffen. Wir haben dennoch erste

Vergleichsanalysen zwischen Tumor- und Normalgewebe am vorliegenden Material durchgeführt, um exemplarisch das Potential unserer Methode darzustellen.

In Rahmen einer *GeneOntology* Analyse konnte so gezeigt werden, dass im Fibroblastenkompartiment der Tumorproben Gene aus der Gruppe der Angiogenese gegenüber normaler Ösophagus-Mukosa vermehrt exprimiert waren. Die Bedeutung der CAFs in der Förderung angiogenetischer Prozesse z.B. durch Stimulation einer VEGF Ausschüttung von Tumorzellen wurde bereits durch frühere Studien beschrieben [156-158]. Die Entstehung neuer Blutgefäße stellt einen essenziellen Bestandteil der Tumorentwicklung dar, um die Versorgung der sich schnell teilenden Zellen zu sichern [158]. Bereits heute kann der VEGFR Antikörper Ramucirumab in palliativen Situation beim EAC zum Einsatz kommen [26]. Auf der Ebene der individuellen Gene fanden wir darüber hinaus eine Hochregulation von CD38 im Immunzellkompartiment des Tumorgewebes. CD38 ist ein u.a. von Lymphozyten, Monozyten und Makrophagen exprimiertes Oberflächenprotein, welchem immunsuppressive Eigenschaften zugeordnet werden. Eine Überexpression in Tumorzellen wurde als potenzieller Mechanismus einer Resistenz gegen PD-1/PD-L1 ICIs detektiert [159-161]. Der für das EAC diskutierte Einsatz einer PD-1/PD-L1 Blockade könnte durch dieses Wissen ergänzt werden und den Einsatz einer kombinierten Blockade mit CD38 Antikörpern nahelegen [159, 162].

Derartige Analysen zeigen den potenziellen Nutzen unserer Methode, wenngleich sie aufgrund der noch geringen Probenzahl nicht für Aussagen über die Tumorbiologie des EACs verwendet werden dürfen. In Zukunft sollte die Bedeutung von Alterationen zwischen Normal- und Tumorgewebe zudem auch mit klinischen Daten der Patienten innerhalb prospektiver Studien analysiert werden, um den Einsatz zielgerichteter Therapieoptionen für das EAC weiter zu evaluieren.

7.2 Biomarker des Immunkompartiments und ihre Dynamik unter neoadjuvanter Therapie und Metastasierung

Durch die bereits vorgestellten, aktuellen Ergebnisse der CheckMate 649, ATTRACTION-4 und KEYNOTE-590 Studien zeigt sich die zunehmende Bedeutung der Immuncheckpoint-Inhibition auch für das EAC [40, 112, 113]. Wir untersuchten daher erstmals ein großes Patientenkollektiv von EACs durch immunhistochemische und RNA-Sonden basierte Verfahren auf das Vorkommen der Immuncheckpoints LAG3, VISTA und IDO und korrelierten ihre Expression mit klinischen Daten. Das krankheitsspezifische und UICC Stadien abhängige Überleben der Patienten kann als Kriterium einer repräsentativen Patientenkohorte interpretiert werden.

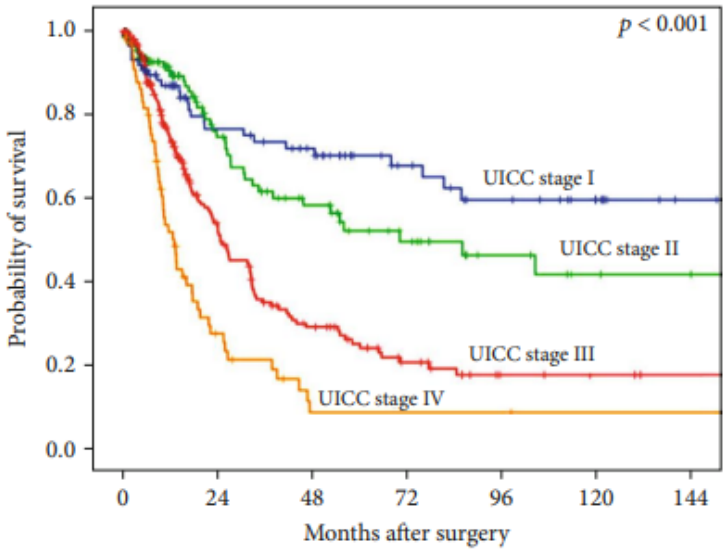

Abbildung 5: (Loeser, Kraemer et al., Journal of Immunology Research, 2020 [163])

UICC Stadien abhängiges Überleben der untersuchten Kohorte

7.2.1 Häufigkeit der Immuncheckpoints LAG3, VISTA und IDO beim EAC

Der Einsatz von ICIs hat die Behandlung einer Vielzahl von soliden Tumoren revolutioniert [101]. Nichtsdestotrotz profitiert nur ein Teil der Patienten von ihrem Gebrauch: So ergeben sich für eine PD-1/PD-L1 Inhibition Ansprechraten von 45,2% beim fortgeschrittenen nicht kleinzelligen Lungenkarzinom (NSCLC), 43,7% beim nicht resektablen malignen Melanom, 13-24% beim fortgeschrittenen Urothelkarzinom und 5-30% beim vortherapierten, triple-negativen Mamma-Karzinom [164-168]. Der tatsächliche Überlebensvorteil ist darüber hinaus oft unbefriedigend: Die vorläufigen Ergebnisse der o.g. Studien beim EAC zeigen beispielsweise einen Vorteil im Gesamtüberleben bzw. progressionsfreien Überleben zwischen 2,2 und 3,3 Monaten

im Vergleich zur Standardtherapie. Die bestehenden Konzepte können dabei zum Teil durch eine gemeinsame Gabe mehrerer ICIs verbessert werden. So zeigt die kombinierte Blockade von PD-1 und CTLA-4 durch Nivolumab und Ipilimumab z.B. beim malignen Melanom eine Verbesserung der Ansprechrate von 43,7% auf 57,6% und beim Mikrosatelliten-instabilen (MSI) Kolonkarzinom von 31% auf 55% [164, 169]. Auch beim EAC wird die duale Blockade von PD-1 und CTLA-4 aktuell im Rahmen einer klinischen Phase II Studie (Radio-Immuno-Chemotherapy of Cancer of the Esophagus, RICE-Studie) untersucht. Die Evaluation weiterer modulierender Immuncheckpoints beim EAC kann daher mögliche Zielproteine einer zukünftigen Immuntherapie offenbaren. Im Folgenden werden unsere Ergebnisse zur Expression der Immuncheckpoints LAG3, VISTA und IDO diskutiert.

Der Immuncheckpoint LAG3 wird auf diversen T-/B-Lymphozyten sowie dendritischen Zellen exprimiert und vermittelt seine immunsuppressiven Eigenschaften über eine Bindung an MHC II Proteine [170]. Wir fanden immunhistochemisch eine Expression auf TILs in 11,4% der EACs in einer Validierungskohorte von 421 Patienten und eine hohe Korrelation mit der entsprechenden mRNA mittels RNA BaseScope Technologie. Verglichen mit Ergebnissen aus kleineren Patientenkollektiven in anderen Tumoren des Gastrointestinaltrakts zeigten wir damit eine etwas geringere Anzahl LAG3 positiver Entzündungszellen, wobei die Daten mitunter stark variieren: So geben Studien beim Magenkarzinom (n=85) eine Expression von 24,7%, beim Kolonkarzinom (n=203) von 23,6%, beim HCC (n=143) von 42% und beim ESCC (zwei Studien mit n=183 bzw. n=287) zwischen 37,7% und 59,9% an [171-174]. Demgegenüber zeigt ein großes Kollektiv von 3992 Patientinnen beim Mammakarzinom 11% LAG3 positive Tumoren, was gut mit unseren Daten übereinstimmt [175]. Kommerzielle Antikörper gegen LAG3 werden derzeit in Kombination mit PD-1 ICIs in klinischen Phase I/II Studien untersucht [176].

Der Immuncheckpoint VISTA zeigt innerhalb des Immunkompartiments eine Expression sowohl auf myeloiden als auch lymphozytären Zellen und wirkt über zumeist noch unbekannte zellintrinsische sowie -extrinsische Signalwege immunsuppressiv auf die Aktivität der T-Lymphozyten [177]. Wir fanden eine vermehrte Expression auf TILs in 29% der 393 Patienten umfassenden Validierungskohorte, wobei eine signifikante Korrelation mit frühen Tumorstadien (T1/T2) auffiel. Immunhistochemische Untersuchungen zu anderen Tumorentitäten

zeigen VISTA Expressionen auf TILs von 16,9% beim HCC, 38,1% beim Pankreaskarzinom und 35,6% beim Ovarialkarzinom [178-180]. Zwei Studien einer Arbeitsgruppe aus Kiel zum Magenkarzinom stellen zudem in Übereinstimmung mit unseren Ergebnissen beim EAC eine signifikante Reduktion der VISTA Expression in höheren Tumorstadien (einmal von T2 zu T3, einmal von T3 zu T4) fest [181, 182]. Erklärungsansätze hierfür sind dabei spekulativ: So ist denkbar, dass insbesondere in frühen, lokal begrenzten Phasen der Tumorentstehung die immunsuppressiven Mechanismen zum „Durchbrechen“ der Immunkontrolle (Übergang von Equilibrium zur Escape-Phase) von besonderer Bedeutung sind. Außerdem könnten besonders aggressive Tumorsubtypen andere Mechanismen des Immune-Escapes in ihrer Progression nutzen. In den beiden genannten Studien zum Magenkarzinom wird darüber hinaus das Vorkommen VISTA positiver Immunzellen mit 83,6% bzw. 98,6% als extrem hoch beschrieben [181, 182]. Dabei wird jedoch bereits das Vorkommen einer einzigen VISTA positiven Immunzelle gewertet, was die Diskrepanz zu unseren Ergebnissen erklärt. Die Erprobung von VISTA Antikörpern ist bislang weniger weit fortgeschritten als die der LAG3-Inhibitoren und befindet sich noch in frühen Phasen der klinischen Forschung [177].

IDO ist ein intrazelluläres Protein, welches durch zahlreiche Immunzellen, aber auch Nicht-Immunzellen (z.B. Tumorzellen) exprimiert wird und den Abbau der essenziellen Aminosäure Tryptophan katalysiert [183]. Eine Expression wird durch IFNγ induziert und durch den Mangel an Tryptophan sowie den zytotoxischen Effekten der Metabolite kommt es in der Folge zur T-Zell Anergie [183]. Wir konnten in 52,6% der Fälle in einer Validierungskohorte von 551 Patienten eine Expression von IDO auf TILs zeigen und dazu passend eine stark korrelierende mRNA-Expression von IFNγ. Genau wie bei VISTA sahen wir eine signifikante Reduktion der Proteinexpression in höheren Tumorstadien (T1/T2 vs. T3/T4). Während für LAG3 und VISTA keine bzw. eine minimale Expression in Tumorzellen (LAG: 0%, VISTA: 1,2%) detektiert wurde, fand sich für IDO eine solche in 9,2% der untersuchten EACs. Interessanterweise existieren in der Literatur fast keine Daten zur Verteilung von IDO explizit auf TILs. Häufig werden dagegen IDO exprimierende Tumorzellen allein oder in Kombination mit TILs evaluiert [184]. Dabei bleibt die Frage offen, ob eine IDO Expression in unterschiedlichen Tumorkompartimenten durch gleiche Funktions- und Induktionsmechanismen beschrieben werden kann oder ob Kompartiment-spezifische Besonderheiten zu beachten sind [184]. So zeigen Zellkulturexperimente, dass eine Tryptophandepletion

im TME durch Tumorzellen mittels Hochregulation von Aminosäuretransportern kompensiert werden kann, während dieser Mechanismus bei T-Lymphozyten fehlt [185]. Eine Blockade von IDO hätte demnach einen ausgeprägteren Effekt auf Immunzellen als auf Tumorzellen [185]. Auch für immunhistochemische Analysen sind daher nach Zelltyp getrennte Auswertungen der Proteinexpression von großer Bedeutung.

Wie relevant eine Beschreibung der Proteinexpression von Immuncheckpoints im klinischen Alltag sein kann, zeigt das Beispiel der Bestimmung von PD-L1 in zahlreichen soliden Tumoren: Therapien mit ICIs gegen PD-1 oder PD-L1 erfordern aufgrund der prädiktiven Bedeutung in der Mehrzahl den immunhistochemischen Nachweis einer erhöhten PD-L1 Expression. Wenngleich die von uns untersuchten Immuncheckpoints bislang klinisch weniger intensiv erforscht sind, könnte die hier gezeigte Proteinexpression in einem relevanten Anteil von EACs somit zur Implementierung von zukünftigen Immuntherapien beitragen. Ungeachtet davon zeigen unsere Ergebnisse den Nutzen von Kompartiment-spezifischen Untersuchungen auch für die immunhistochemische Analyse von Immuncheckpoints, deren Bedeutung in unterschiedlichen Tumorkompartimenten somit besser bestimmt und verstanden werden kann.

7.2.2 Diagnostischer Nutzen bioptisch gewonnener Gewebeproben zur Evaluierung der Immuncheckpoints LAG3, VISTA und IDO beim EAC

Die genaue Bestimmung der Proteinexpression stellt aufgrund der beschriebenen klinischen Konsequenz einen wichtigen Bestandteil in der Untersuchung von Immuncheckpoints dar. Da beim EAC in der initialen Diagnostik meist endoskopisch Biopsien entnommen werden, stellt sich die Frage, inwieweit diese repräsentativ für den gesamten Tumor sind. Um dies für die Immuncheckpoints LAG3, VISTA und IDO zu beantworten, entwarfen wir einen multi-spot-Tissue-Microarray, auf welchem bis zu acht unterschiedliche Gewebeproben aus zwei unterschiedlichen Lokalisationen eines Tumors abgebildet wurden (vier aus der endoluminalen Tumoroberfläche und vier aus dem tiefer gelegenen Infiltrationsrand). Damit konnten wir die etwaige Heterogenität der Immuncheckpoint-Expression innerhalb einer Lokalisation (oberflächlich bzw. tief) evaluieren und gleichzeitig Unterschiede zwischen endoluminal gelegenen Proben und dem Infiltrationsrand beschreiben.

Wir fanden für alle drei Immuncheckpoints in einem Großteil der Fälle ein heterogenes Verteilungsmuster innerhalb der vier Proben einer Lokalisation. Zur leichteren Auswertung fassten wir die jeweilige Expression in Form von Mittelwerten für die weitere Auswertung zusammen. Ein Vergleich zwischen den Mittelwerten der Tumoroberfläche und dem Infiltrationsrand ergab eine extrem starke Korrelation zwischen den zwei unterschiedlichen Lokalisationen. Es ist daher anzunehmen, dass bioptisch gewonnene Gewebeproben der Tumoroberfläche, bezogen auf die Expression von LAG3, VISTA und IDO, ein verlässliches Bild für den gesamten Tumor beim EAC abgeben können. Gleichzeitig scheint es sinnvoll, mehrere Biopsien zu analysieren, um die etwaige Heterogenität innerhalb des Tumors zu erfassen [186].

7.2.3 Prognostische Bedeutung der Immuncheckpoints LAG3, VISTA und IDO und ihre therapeutische Konsequenz beim EAC

Entgegen der initialen Hypothese fanden wir für alle drei untersuchten Immuncheckpoints bei Betrachtung der jeweiligen Validierungskohorte eine signifikant günstige Bedeutung für die Prognose beim EAC. Subgruppenanalysen zeigten dabei, dass bei LAG3 der Prognosevorteil in der Gesamtkohorte allein auf fortgeschrittenen Tumorstadien (T3/T4) basierte (keine Relevanz in der T1/T2 Subgruppe). Bei VISTA sahen wir umgekehrt eine prognostische Relevanz ausschließlich in frühen Tumorstadien als Treiber der Prognose des gesamten Patientenkollektivs. Bei IDO war der Effekt stadienunabhängig und in beiden Subgruppen vorhanden. Aus diesen Resultaten ergaben sich zwei Fragen: 1) Warum korreliert das Vorkommen immunsuppressiver Immuncheckpoints mit einer günstigen Prognose? 2) Welchen Stellenwert hat die prognostische Bedeutung von Immuncheckpoints beim EAC?

Der prognostisch günstige Effekt einer verstärkten Expression der untersuchten Immuncheckpoints wirkt zunächst kontraintuitiv: Eine durch sie reduzierte Immunantwort würde entsprechend dem Modell zum Immunoediting nach Schreiber erwartungsgemäß mit einem früheren Entkommen der Tumorzellen aus der Kontrolle des Immunsystems und damit einer schlechteren Prognose einhergehen [80]. Einen alternativen Erklärungsansatz liefert daher die Hypothese, dass die Expression z.B. von LAG3, IDO und VISTA auch als Ausdruck einer starken Immunantwort auf einen besonders immunogenen Tumorphänotypen gewertet werden kann: Dabei stellt die Hochregulation von immunsuppressiven Immuncheckpoints ein kompensatorisches Element in einem hoch inflammatorischen TME dar. Dies entspricht einer Theorie von

Llosa et al., welche in der prognostisch günstigen und besonders immunogenen Subgruppe der MSI kolorektalen Karzinome eine erhöhte Proteinexpression von PD-1, PD-L1, CTLA-4, LAG-3 und IDO zeigen [187]. In dem von uns untersuchten Patientenkollektiv fanden wir für LAG3 und IDO eine signifikante Korrelation mit dem Vorkommen von CD3 positiven TILs. Darüber hinaus zeigte sich für IDO eine Korrelation mit der mRNA Expression des proinflammatorischen Zytokins IFNγ. Das vermehrte Vorkommen von TILs ist für zahlreiche Tumorentitäten, u.a. auch für das EAC, mit einem verlängerten Überleben korreliert [188, 189]. Es scheint daher naheliegend, dass der prognostisch günstige Effekt der untersuchten Immuncheckpoints vielmehr Ausdruck einer starken Immunreaktion als eine direkte Folge der Immuncheckpoints selbst ist. Dennoch müssen auch Checkpoint-individuelle Erklärungsansätze für den prognostisch günstigen Effekt berücksichtigt werden: So scheint für ggf. VISTA ein inflammationsunabhängiger Mechanismus zu existieren, da trotz günstiger Prognose keine Korrelation mit CD3 positiven Zellen gezeigt werden konnte. Da der Wirkmechanismus weitgehend unbekannt ist, könnten auch andere Effekte neben der reinen Suppression der T-Lymphozyten Aktivität hierbei eine Rolle spielen [177]. Für IDO könnten zudem auch die potenziell schädliche Wirkung der Tryptophandepletion auf die Tumorzellen oder der anti-tumorale Effekt von IFNγ die Prognose günstig beeinflussen [190].

Ungeachtet dieser Überlegungen muss der Stellenwert von Immuncheckpoints zur Prognoseabschätzung hinterfragt werden. Auf der einen Seite haben in unseren Untersuchungen IDO- und VISTA-exprimierende, frühinvasive Tumore (T1/T2) aufgrund ihrer enormen Vorteile im medianen Überleben (VISTA: 202,2 Monate vs. 21,6 Monate, IDO: 142,1 vs. 37,1 Monate) das Potential, Patientengruppen zu definieren, welche aufgrund ihrer ohnehin guten Prognose weniger von neoadjuvanten Therapiekonzepten profitieren könnten als andere. Sollten prospektive Studien unsere Ergebnisse belegen, könnte als klinische Konsequenz daraus in Zukunft diesen Patienten z.B. eher zu einer primären Operation geraten werden. Auf der anderen Seite bleibt festzuhalten, dass die Beurteilung der prognostischen Bedeutung von Immuncheckpoints in der Literatur höchst inkonsistent ist: Eine Metaanalyse zum bislang am intensivsten erforschten Immuncheckpoint (-Ligand) PD-1/PD-L1 zeigt beispielsweise für Magen- und Ösophaguskarzinome bei 25 eingeschlossenen Studien 15 Mal einen negativen prognostischen Wert, 5 Mal keinen Einfluss und 5 Mal einen positiven prognostischen Wert einer verstärkten PD-L1 Expression [191]. Ein

ähnlich heterogenes Bild zeigt sich auch für die von uns untersuchten Immuncheckpoints LAG3, VISTA und IDO [99, 175, 178, 190, 192-198]. In nachvollziehbarer Weise spielt die Expression von Immuncheckpoints in Bezug auf die Prognoseabschätzung angesichts dieser Unsicherheit bislang für keine Tumorentität im klinischen Alltag eine Rolle. In Zukunft sind daher weitere Studien an großen Patientenkohorten notwendig, um auch beim EAC ein deutlicheres Bild von einer möglichen Bedeutung der Immuncheckpoints als prognostische Biomarker zu erhalten.

7.2.4 Einfluss von neoadjuvanter Therapie und Metastasierung auf das Immunkompartiment

Der Einsatz von neoadjuvanten Therapien spielt in der Behandlung des lokal fortgeschrittenen EACs eine entscheidende Rolle [26]. Während der Effekt einer (Radio-) Chemotherapie auf das TME u.a. beim Rektumkarzinom als gesichert gilt, ist die Datenlage hierfür beim EAC ausgesprochen limitiert und auch in anderen Tumorentitäten werden entsprechende Fragegestellungen kontrovers diskutiert [116, 118-122]. Die zusätzliche Gabe eines ICIs zu neoadjuvanten Therapiekonzepten wird aktuell in zahlreichen Studien untersucht, so etwa beim EAC die Gabe des PD-L1 Inhibitors Durvalumab zum CROSS Schema im Rahmen der RICE Studie [199]. Das Ansprechen auf ICI-Therapien hängt dabei jedoch von den Eigenschaften des Immunkompartiments ab [107]. Um den Einfluss einer neoadjuvanten Therapie auf das Immunkompartiment beschreiben zu können, untersuchten und verglichen wir deshalb mittels der NanoString® Technologie die Genexpressionsdaten immunologisch relevanter Gene in therapienaiven und neoadjuvant vorbehandelten Gewebeproben. Für Letztere berücksichtigten wir einerseits Proben aus Ösophagusgewebe (Primärtumor) und andererseits solche aus hämatogenen Metastasen. Die Ergebnisse werden im Folgenden diskutiert.

In vorbehandeltem Primärtumor-Gewebe fanden wir einen unterschiedlichen Effekt der (Radio-) Chemotherapie auf bekannte Immuncheckpoints: Während CTLA-4 und PD-L1 durch diese nicht beeinflusst wurden, zeigte sich eine signifikante Reduktion der bereits diskutierten Immuncheckpoints IDO und LAG3 in den Operationspräparaten nach neoadjuvanter Therapie. Derartige Erkenntnisse müssen in der Planung von zukünftigen ICI-Therapien berücksichtigt werden, da eine Herunterregulation der Zielproteine die Effektivität einer Therapie negativ beeinflussen könnte. Darüber

hinaus zeigte sich auch eine signifikante Reduktion in der Genexpression zahlreicher, weiterer Biomarker des adaptiven Immunsystems: So fanden wir u.a. eine Reduktion des T-Zell Markers CD3, was auf eine verminderte Anzahl an TILs in neoadjuvant vortherapiertem Gewebe hindeutet. Auch dies ist mit einem geringeren Ansprechen auf ICI-Therapien assoziiert und verdeutlicht den potenziell immunsuppressiven Effekt einer neoadjuvanten Therapie auf das TME [107].

Damit übereinstimmend finden Park et al. in Operationspräparaten von neoadjuvant vorbehandelten Mammakarzinomen ebenfalls reduzierte Mengen an TILs [119]. Zudem zeigen sie eine Reduktion weiterer immunstimulierender Zelltypen sowie eine erhöhte Fraktion immunsuppressiver Zellen wie M2 Makrophagen, passend zu einem insgesamt immunsupprimierten TME. Ergänzend betrachtet ihre Studie neben dem therapienaiven und postneoadjuvanten Zustand des Operationspräparats jedoch noch einen dritten, dazwischenliegenden Zeitpunkt. Dieser liegt etwa drei Wochen nach dem ersten Chemotherapie-Zyklus. Hier zeigt sich in den Gewebeproben interessanter Weise ein signifikant erhöhtes Level von TILs und eine vermehrte Expression zahlreicher, inflammatorischer Gene im Vergleich zu den beiden anderen Zeitpunkten. Dies deutet darauf hin, dass neoadjuvante Therapien durch einen zu Beginn stark inflammatorischen Effekt eventuell ein zeitliches Fenster für den optimalen Einsatz von ICIs schaffen könnten. Dieses könnte, wie im Falle der hier vorgestellten Studie von Park et al., zum Zeitpunkt der Operation aufgrund eines dann immunsupprimierten TMEs bereits nicht mehr nachweisbar sein. Die alleinige Betrachtung von Operationspräparaten, wie sie u.a. auch in der von uns durchgeführten Studie erfolgte, kann daher wahrscheinlich nur einen Teil des dynamischen Effekts von neoadjuvanten Therapien auf das TME abbilden. Es scheint von Bedeutung zu sein, dass zukünftige Studien beim EAC den Einfluss von (Radio-) Chemotherapie auch im zeitlichen Verlauf evaluieren. Die Umsetzung derartiger Studien könnte sich dabei aufgrund der hohen Invasivität endoskopisch gewonnener Proben jedoch schwierig gestalten.

Darüber hinaus konnten wir im Rahmen unserer Untersuchungen an Operationspräparaten des Primärtumors zeigen, dass die Expression von PD-L1 (unabhängig vom Einfluss der Neoadjuvanz) im Vergleich zu anderen Immuncheckpoints wie LAG3, TIM-3, CTLA4 und CD276 deutlich geringer ausgeprägt war. Dies unterstützt den Ansatz einer kombinierten Anwendung von ICIs mit unterschiedlichen Zielproteinen über die PD-1/PD-L1 Blockade hinaus.

Unsere Daten zur Immunmodulation in hämatogenen Metastasen des EACs befinden sich derzeit im Review („*Gene expression changes in metastatic esophageal adenocarcinoma"* von Wagener, Kraemer et al., submitted to Cancers, Basel), so dass an dieser Stelle lediglich ein kurzer Ausblick auf wesentliche Erkenntnisse dieses Projekts erfolgen soll. So ergaben sich Unterschiede in der Expression immunmodulatorisch wirksamer Gene zwischen unbehandeltem EAC Gewebe des Ösophagus und hämatogenen Metastasen: Diese zeigten sich u.a. in signifikanten Veränderungen bei der Expression von Interleukinen und deren Rezeptoren (z.B. IL24, IL1RN), Chemokinen und deren Rezeptoren (z.B. CXCL5, CXCR4), Komplementfaktoren (z.B. C7), Phagozytose-relevanter Rezeptoren auf Makrophagen (z.B. MARCO) und B-Lymphozyten-Markern (z.B. TNFRSF1). Veränderungen in der Expression der klinisch bedeutsamen Immuncheckpoints nach neoadjuvanter Therapie konnten wir dagegen mit Ausnahme einer Hochregulation von TIM-3 in Lungenmetastasen nicht feststellen. Wenngleich somit eine klinische Relevanz der Immunmodulation in hämatogenen Metastasen EAC unklar bleibt, so zeigen unsere vorläufigen Ergebnisse, dass der Status von Immunmarkern im unbehandelten Primärtumor nicht zwangsläufig auf hämatogene Metastasen übertragen werden kann. Lokal unterschiedliche Adaptionsmechanismen und der bereits zuvor beschriebene Effekt einer (Radio-) Chemotherapie führen offensichtlich zu einer Modulation in der Immunantwort auf die Tumorzellen. Dabei bleibt festzuhalten, dass Untersuchungen zur Genexpression an metastatischem Gewebe aufgrund der zumeist fehlenden Indikation zur Resektion auf sehr geringen Patientenzahlen beruhen. So konnten wir beispielsweise für das oben genannte Projekt lediglich Material aus 10 Primärtumoren und 10 hämatogenen Metastasen gewinnen, so dass weitere Studien zur Bestätigung unserer vorläufigen Ergebnisse notwendig sind.

Zusammenfassend konnten wir durch unsere Untersuchungen einen immunsuppressiven Effekt einer neoadjuvanten Therapie auf das Immunkompartiment in Operationspräparaten des Ösophagus nachweisen und fanden Hinweise für eine Immunmodulation in vortherapierten, hämatogenen Metastasen mit nicht eindeutig geklärter klinischer Relevanz. Die Frage bleibt offen, ob die einzeitige Evaluation des Immunstatus z.B. an Operationspräparaten ein zuverlässiges Bild der wohl dynamischen Wirkung einer (Radio-) Chemotherapie auf das TME abgeben kann. Ggf. könnten mehrzeitige Untersuchungen mittels endoskopischer „deep biopsies" bei dieser Fragestellung in Zukunft weiterhelfen. Zudem muss beachtet werden, dass

aufgrund der Gewinnung der RNA aus Formalin-fixiertem Material auch keine differenzierte Unterscheidung der Genexpression nach unterschiedlichen Zelltypen erfolgen konnte. Der Anteil der jeweiligen Tumorkompartimente an den diskutierten Ergebnissen bleibt daher unklar: Beispielsweise kann die festgestellte Reduktion der IDO Expression in Ösophagus-Resektaten durch eine Reduktion in Tumorzellen, aber auch in Immunzellen begründet werden. Einerseits wäre dies somit Ausdruck einer effektiven, zytotoxischen Wirkung der neoadjuvanten Therapie auf die Tumorzellen, andererseits Teil eines supprimierenden Effekts auf Immunzellen. Kompartiment-spezifische Transkriptomanalysen von therapienaiven und neoadjuvant vorbehandelten Patienten sollten daher in Zukunft den Einfluss der Therapie auf das TME beim EAC näher charakterisieren.

7.3 Biomarker in ErbB-Rezeptor abhängigen Signalwegen im Tumorzellkompartiment

7.3.1 Häufigkeit und prognostische Bedeutung von Amplifikationen in ErbB-Rezeptor abhängigen Signalwegen

Im Tumorzellkompartiment sind Alterationen in ErbB-Rezeptor abhängigen Signalwegen häufig Angriffspunkte einer zielgerichteten Therapie [200]. Dabei spielen insbesondere CNVs eine wichtige Rolle beim EAC und TCGA Daten können bedeutsame Amplifikationen in diesem Signalweg belegen [139, 140, 201, 202]. Wir untersuchten daher mittels FISH-Technik und Immunhistochemie an einem großen Patientenkollektiv mit EAC die Verteilung und prognostische Bedeutung von HER2/neu, KRAS und PIK3CA Amplifikationen und ihrer Proteinexpression.

Dabei konnten wir Amplifikationen von KRAS in 17,1%, PIK3CA in 5% und eine HER2/neu Überexpression in 14,9% der über 400 untersuchten EACs nachweisen. Insbesondere HER2/neu stellt einen bereits ausführlich untersuchten Biomarker des EACs dar: Literaturdaten zeigen zu unseren Ergebnissen vergleichbare Raten einer Überexpression des Proteins zwischen 11 und 29% [147, 148, 203-207]. Diese kann einerseits durch Genamplifikationen, aber auch durch andere Mechanismen wie eine Polysomie des Chromosoms 17, aktivierende Mutationen oder epigenetische und posttranskriptionale Veränderungen zustande kommen, so dass die Amplifikation nicht unbedingt mit der Proteinexpression übereinstimmen muss [208]. TCGA Daten, erhoben an 87 EACs, beschreiben Amplifikationen von HER2/neu (ErbB2) in 28,7%

der Fälle, was die enorme Relevanz für diese Tumorentität unterstreicht [139, 140]. Zum Vergleich: Beim Mammakarzinom, einem bedeutendem Vertreter HER2/neu positiver Malignome, zeigen die Daten von 1070 Patientinnen Amplifikationen in 11,5% der Fälle [139, 140]. Darüber hinaus fanden wir KRAS Amplifikationen in einer etwas höheren Frequenz als in den TCGA Daten gezeigt (dort 9,2%) [139, 140]. Unsere Ergebnisse bestätigen jedoch eine große Studie von Wong et al. zu Tumoren des gastroösophagealen Übergangs (ebenfalls 17%) [143]. Für PIK3CA stimmten unsere Daten mit den TCGA Ergebnissen überein (jeweils in etwa 5% amplifiziert) [139, 140]. Interessanterweise fanden wir zudem eine signifikante Korrelation zwischen dem HER2/neu und KRAS Amplifikationsstatus. Während KRAS Mutationen einen gesicherten Resistenzmechanismus einer HER2/neu Blockade durch den Antikörper Trastuzumab darstellen, wird ein ähnlicher Effekt auch für KRAS Amplifikationen vermutet [209, 210]. Da ausschließlich HER2/neu positive Patienten beim EAC mit Trastuzumab behandelt werden, könnten diese Ergebnisse verringerte Ansprechraten für doppelt-amplifizierte Patienten erklären.

Bezogen auf die klinischen Daten der Patienten fanden wir lediglich für die HER2/neu Überexpression/Amplifikation einen signifikanten und prognostisch günstigen Effekt in der Gesamtkohorte. HER2/neu positive Tumore zeigten zudem eine Korrelation mit frühen Tumorstadien (T1/T2) und dem Fehlen von Lymphknotenmetastasen. Viele Studien mit Überlebensdaten unterscheiden die Adenokarzinome des oberen Gastrointestinaltrakts nicht weiter, so dass die Datenlage von vergleichbaren Studien für das reine EAC begrenzt und zudem kontrovers diskutiert ist. Die derzeit größte Studie zum EAC von Yoon et al. bestätigt jedoch unsere Ergebnisse und zeigt bei 713 Patienten mit einem operablen EAC ebenfalls einen signifikanten Überlebensvorteil für HER2/neu überexprimierende Tumore [147]. Für KRAS konnten wir darüber hinaus in der Subgruppe der therapienaiven, primär-operierten Tumore eine negative prognostische Bedeutung der Amplifikation erkennen. Aufgrund der nur knapp erreichten Signifikanz (p=0,05) und der limitierten Zahl primär operierter, KRAS amplifizierter Patienten (n=41), muss dieser potenziell aggressivere EAC-Subtyp in zukünftigen Studien weiter evaluiert werden.

7.3.2 Veränderungen im Immunkompartiment von PIK3CA amplifizierten Tumoren

Interessanterweise fanden wir eine Anreicherung von PIK3CA amplifizierten Tumorzellen in T-Lymphozyten reichen Tumoren (11,9% in CD3 reichen vs. 3 % in CD3 armen Tumoren). Es handelt sich hierbei um ein bisher nicht beschriebenes Phänomen und ein Beispiel für die potenzielle Interaktion von Immun- und Tumorzellkompartiment. Das Vorkommen von PIK3CA Amplifikationen ist darüber hinaus bereits auch in Immunzellen beschrieben: Hier scheint sie eine Bedeutung für den Verlauf inflammatorischer Erkrankungen zu haben [211, 212]. Erklärungen für den Zusammenhang zwischen PIK3CA Amplifikationen in Tumorzellen und einer vermehrten Inflammation sind dagegen spekulativ. Beim Ovarialkarzinom konnte eine Hochregulation von PIK3CA in Tumorzellen durch den Transkriptionsfaktor NF-κB nachgewiesen werden [213]. NF-κB kann wiederum u.a. durch proinflammatorische Zytokine vermehrt aktiviert werden [214]. Dies würde einen Mechanismus darstellen, in dem durch Inflammationsprozesse das Tumorwachstum über die Aktivierung proliferativer Signalwege begünstigt wird. Andersherum wäre es jedoch auch denkbar, dass PIK3CA amplifizierte Tumore einen besonders immunogenen Tumorphänotypen darstellen. Dieser könnte daher beispielsweise verstärkt von ICI-Therapien profitieren. In dem von uns untersuchten Patientenkollektiv konnten wir jedoch keine Korrelation von PIK3CA Amplifikationen mit der Expression von Immuncheckpoints nachweisen, so dass dieser Subtyp vermutlich andere Strategien zur Immunevasion nutzt. Die mögliche Interaktion verschiedener Tumorkompartimente ist ein weiterer Beleg dafür, wie Kompartiment-spezifische Analysemethoden zu einem besseren Verständnis der Tumorbiologie beitragen. So könnte z.B. die vermehrte Genexpression NF-κB aktivierender Zytokine im Immunzellkompartiment auf die Induktion einer PIK3CA Amplifikation in Tumorzellen hindeuten. Dies würden wichtige Hinweise für den genauen Mechanismus unserer Beobachtungen liefern.

8. Zusammenfassung

Das ösophageale Adenokarzinom zeigt eine global steigende Inzidenz und hat mit einer 5-Jahres-Überlebensrate von weniger als 25% eine sehr schlechte Prognose. Personalisierte Therapieansätze sind bislang auf den Einsatz von HER2/neu Inhibitoren begrenzt und prognostisch oder prädiktiv relevante Biomarker des Tumormikromilieus sind unzureichend charakterisiert. Die vorliegenden Publikationen, welche die Grundlage der Dissertation darstellen, nähern sich dieser Problematik durch drei unterschiedliche Schwerpunkte.

1. Zur Identifizierung Kompartiment-spezifischer Biomarker wurde eine Methode entwickelt, welche als Alternative zum Single Cell Sequencing Genexpressionsprofile individueller Zelltypen generieren kann. Dabei erfolgt die Extraktion der RNA nicht aus Einzelzellen, sondern aus flowzytometrisch-getrennten Zellkompartimenten des EAC-Gewebes. Wir validierten für unser Verfahren eine erfolgreiche Separation der Proben in Epithelzellen, Immunzellen und Fibroblasten und demonstrierten eine suffiziente Ausbeute an RNA-Material auch für kleine Gewebemengen aus bioptisch gewonnenen Proben. Hierdurch können therapienaive EAC-Biopsien, welche im Rahmen des Primärstagings routinemäßig entnommen werden, im Vergleich zu gesunder Ösophagus-Schleimhaut Kompartiment-spezifisch sequenziert werden. Im Gegensatz zum Single Cell Sequencing entstehen durch unser Verfahren deutlich geringere Kosten, so dass eine Anwendung auch in größeren Patientenkohorten ermöglicht wird. Zukünftig können damit im Rahmen weiterführender Studien prognostische und prädiktive Biomarker im Tumormikromilieu des EACs detektiert werden.

2. Konkrete Biomarker des Immunzellkompartiments wurden darüber hinaus in einem großen Patientenkollektiv von bis zu 551 Patienten auf ihre Bedeutung beim EAC hin überprüft. Wir konnten dabei eine Expression der Immuncheckpoints LAG3, VISTA und IDO auf TILs durch immunhistochemische und RNA-Sonden basierte Verfahren in einem relevanten Anteil der Tumore nachweisen (LAG3: 11,4%, VISTA: 29%, IDO: 52,6%). Als Zielproteine von Immuncheckpoint-Inhibitoren wird ihr therapeutischer Stellenwert bei anderen Tumorentitäten derzeit in klinischen Studien evaluiert. Auch beim EAC verdeutlichen erste Ergebnisse aus aktuellen Phase III Studien den potenziellen Nutzen der Immuncheckpoint-Inhibitoren. Der Nachweis der o.g. Proteine beim EAC deutet daher auf therapeutische Angriffspunkte im Immunzellkompartiment

hin. Zudem konnte eine prognostisch günstige Bedeutung der VISTA, LAG3 und IDO Expression nachgewiesen werden. Wenngleich dieser Effekt wahrscheinlich auf ein stark inflammatorisches Mikromilieu dieser Tumoren zurückzuführen ist, könnten die enormen Überlebensvorteile z.B. in der Subgruppe frühinvasiver, IDO oder VISTA exprimierender Tumore zukünftige Therapieentscheidungen beeinflussen. Weiterhin wurde durch den Vergleich von immunogenen Genexpressionsprofilen aus therapienaiven und vorbehandelten Tumoren mittels der NanoString® Technologie ein immunsuppressiver Effekt von neoadjuvanten Therapiekonzepten auf das lokale Tumormikromilieu in Operationspräparaten des EACs gezeigt. Dabei kam es u.a. zur verminderten Expression von Immuncheckpoints und einer geringeren Anzahl Tumor infiltrierender Lymphozyten nach (Radio-) Chemotherapie. Diese Ergebnisse sollten bei der derzeit im Rahmen klinischer Studien untersuchten, kombinierten Gabe von Immuncheckpoint-Inhibitoren und konventionellen Therapien berücksichtigt werden. Die klinische Relevanz der darüber hinaus festgestellten Immunmodulation in hämatogenen Metastasen muss aufgrund der geringen Patientenzahl in Zukunft weiter evaluiert werden.

3. Im Tumorzellkompartiment wurde weiterhin die Rolle von Amplifikationen in ErbB-Rezeptor abhängigen Signalwegen durch FISH-Technik und Immunhistochemie evaluiert. Die Bedeutung von Copy Number Variations als Biomarker beim EAC wurde bereits durch frühere Studien beschrieben und konnte durch unsere Untersuchungen bestätigt werden. So fanden wir KRAS Amplifikationen in 17,1%, PIK3CA Amplifikationen in 5% sowie eine HER2/neu Protein-Überexpression in 14,9% der untersuchten Tumore. Eine HER2/neu Überexpression war darüber hinaus mit einem verbesserten Überleben der Patienten mit einem operablen Ösophaguskarzinom korreliert. PIK3CA amplifizierte Tumore fanden sich zudem signifikant angereichert in T-Lymphozyt-reichen Tumoren, was auf eine mögliche Interaktion von Tumor- und Immunkompartiment hindeuten könnte.

Insgesamt sollen die Ergebnisse dieser Arbeit zur Entwicklung personalisierter Therapieoptionen beim EAC beitragen. Das entwickelte Verfahren zur Sequenzierung von flowzytometrisch getrennten EAC-Biopsien ermöglicht die Kompartiment-spezifische Detektion potenzieller Biomarker im Tumormikromilieu. Darüber hinaus treten bereits aus anderen Tumorentitäten bekannte Immuncheckpoint-Proteine sowie

Genamplifikationen auch beim EAC in einem relevanten Anteil der Tumore auf und repräsentieren somit mögliche Angriffspunkte zukünftiger Therapiestrategien.

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10. Abbildungsverzeichnis

11. Lebenslauf

Persönliche Daten

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Telefon	0176 41541205
E-Mail-Adresse	maxkraemer94@web.de
Geburtsdatum / -ort	31.01.1994 in Münster
Staatsangehörigkeit	deutsch
Familienstand	ledig

Ausbildung

07/2013	Abitur am Gymnasium St. Mauritz in Münster	Note: 1,0
10/2014-05/2021	Studium der Humanmedizin an der Universität zu Köln	
	10/2016: Äquivalenzprüfung (Physikum)	Note: gut (1,63)
	04/2020: 2. Staatsexamen	Note: gut (2,0)
	05/2021: 3. Staatsexamen	Note: sehr gut (1,0)

Praktische Erfahrungen

Famulaturen

07/2017	Famulatur in der Hausarztpraxis Marienburg (4 Wochen)
07/2018	Famulatur in der Klinik III für Innere Medizin, Kardiologie, Uniklinik Köln: Normalstation 3.1 (2 Wochen) und kardiologische IMC Station (2 Wochen)
03/2019	Famulatur im Institut für Pathologie, Uniklinik Köln (4 Wochen)
08/2019	Famulatur in der Pädiatrie, Uniklinik Köln: Station Kinderonkologie (2 Wochen)
08/2019	Famulatur in der Klinik I für Innere Medizin, Hämatologie/Onkologie, Uniklinik Köln: Station 15.1 A (2 Wochen)

Praktisches Jahr

05/2020 – 09/2020	1. Tertial: Innere Medizin Klinik I für Innere Medizin, Hämatologie/Onkologie, Uniklinik Köln: Station 16.2 B (8 Wochen) und Station 11.2 (8 Wochen)
09/2020-12/2020	2. Tertial: Anästhesiologie Radboud Universitätsklinikum, Nijmegen (NL) (8 Wochen) und Uniklinik Köln (8 Wochen)

12/2020-04/2021	3. Tertial: Chirurgie Klinik für Gefäßchirurgie, Klinik für Viszeralchirurgie, Klinik für Herz-Thoraxchirurgie, Uniklinik Köln

Stipendien

Seit 01/2015	Studienförderung durch die Studienstiftung des Deutschen Volkes
11/2018-10/2019	Promotionsstipendium „Köln Fortune“ durch die Universität zu Köln

Außeruniversitäres Engagement

08/2013-07/2014	Bundesfreiwilligendienst im Pflegedienst auf der onkologischen Normalstation der Uniklinik Münster
07/2015-11/2017	aktives Mitglied als Betreuer einer Fußballmannschaft im Verein „Grenzenlos in Bewegung“ zur Flüchtlingshilfe, initiiert durch die Sporthochschule Köln

Weitere Nebentätigkeiten

10/2017-07/2019	Studentischer Mitarbeiter in der Hausarztpraxis Marienburg in der Versorgung von Patienten im Praxisalltag

Sprachkenntnisse

Deutsch als Muttersprache

Englisch fließend in Schrift und Sprache

Niederländisch gute Kenntnisse in Wort und Schrift