Aus dem CharitéCentrum 14 – Tumormedizin
Klinik für Hämatologie und Onkologie
Campus Benjamin Franklin
Ärztlicher Leiter: Prof. Dr. med. A. Pezzutto

Habilitationsschrift

Molekulare Charakterisierung der T-Zell akuten lymphoblastischen Leukämie zur Therapieoptimierung

zur Erlangung der Lehrbefähigung für das Fach

Innere Medizin

**vorgelegt dem Fakultätsrat der Medizinischen Fakultät
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von

Dr. med. Martin Neumann

aus Hannover

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Dekan: Prof. Dr. med. Axel R. Pries
1. Gutachter: Prof. Dr. Martin Schrappe, Kiel
2. Gutachter: Prof. Dr. Dr. Michael Kneba, Kiel

INHALTSVERZEICHNIS

1. EINLEITUNG

Akute lymphoblastische Leukämien (ALL) im Erwachsenenalter stellen immer noch eine therapeutische Herausforderung dar. Während im Kindesalter über 80% aller Patienten geheilt werden können und im Rezidiv der Erkrankung immer noch kurative Therapieoptionen bestehen, ist die Prognose für erwachsene Patienten deutlich schlechter.

Auch wenn die Prognose in den letzten Jahren durch risikostratifizierte Therapieschemata verbessert werden konnte, rezidivieren etwa 50% aller Patienten in den ersten Jahren nach initialer Therapie. In der Rezidivsituation sind kurative Therapieoptionen limitiert.

Neue, zielgerichtete Therapien, wie der monoklonale CD20-Antikörper Rituximab, Tyrosinkinase-Inhibitoren (TKI) oder der bi-spezifische Antikörper Blinatumomab haben in der B-Vorläufer ALL (B-cell precursor-ALL, BCP-ALL) bereits Therapien verändert und versprechen für die Zukunft einen größeren therapeutischen Handlungsspielraum, auch in Rezidivsituationen. Für die T-Zell akute lymphoblastische Leukämie (T-ALL) fehlen solche zielgerichtete Therapieoptionen allerdings bislang. Daher kommt der Identifizierung prognostischer Marker für eine initiale Therapieintensivierung sowie der Identifizierung potentieller therapeutischer Zielstrukturen in der T-ALL eine entscheidende Rolle zu.

Hierzu werden in dieser Arbeit verschiedene Strategien verfolgt. Zum einen wird die im Moment auf dem Immunphänotyp basierende Risikostratifizierung am Beispiel der Subgruppe der „early-T-cell-precursor“ (ETP)-ALL weiter verfeinert. Über die umfassende molekulare und klinische Untersuchung werden Charakteristika deutlich, die von der Standardtherapie abweichende Therapien ermöglichen und somit im Besonderen im Rezidiv dieser Hochrisikoerkrankung zusätzliche Therapieoptionen bieten.

Aber auch innerhalb der immunphänotypisch definierten Subgruppen ist der Krankheitsverlauf der T-ALL sehr heterogen. Eine bessere prognostische Abschätzung mag sich durch die Einbeziehung von Genexpressionen von bestimmten Kandidatengenen ergeben. Dies wird exemplarisch für den Transkriptionsfaktor *BCL11B*, der eine entscheidende Rolle in der T-Zell-Entwicklung besitzt, gezeigt. Da dieser auch regelmäßig in der T-ALL mutiert ist, ergeben sich über die prognostische Bedeutung hinaus auch funktionelle Ansätze für eine zielgerichtete Therapie.

Durch die technischen Entwicklungen der letzten Jahre mit der Möglichkeit der umfassenden Parallelsequenzierung, ist die umfassende molekulare Charakterisierung eines individuellen Patienten keine Zukunftsmusik mehr. Für die T-ALL im Allgemeinen und die ETP-ALL im Speziellen wird im Folgenden untersucht, wie durch Exomsequenzierung und Genpanelsequenzierung Alterationen identifiziert werden können und mit Signalwegaktivierungen in Verbindung gebracht werden können. Die Umsetzung dieser Beschreibungen in therapeutische Strategien wird für die Verbesserung der Prognose der T-ALL des Erwachsenen entscheidend sein.

2. T-ZELL AKUTE LYMPHOBLASTISCHE LEUKÄMIE

2.1 *Modellcharakter der ALL*

Die ALL stellt unbehandelt eine zum Tode führende Erkrankung dar. Bis in die späten 1960er Jahre war die Diagnose einer ALL, dem häufigsten Malignom im Kindesalter, ohne kurative Therapieoption. Heute besitzt die Entwicklung hin zu einer kurativen Therapie der ALL mit einer bis zum heutigen Tag stetigen Verbesserung der Heilungsraten einen Modellcharakter für die Behandlung onkologischer und hämatologischer Neoplasien. Es war die Erkrankung der ALL, in der als erstes eine zytotoxische Therapie mit Aminopterin, einem Folsäureantagonisten, zu ersten dokumentierten Ansprechen führte (Farber *et al*, 1948). Die ALL war auch ein Vorläufer, was Therapien mit Kombinationen von Zytostatika im Rahmen aufeinander aufbauender Studien betrifft. Hier sind vor allem die „total therapies“ des St. Jude Research Hospitals sowie die ALL-BFM (Berlin, Frankfurt, Münster)-Studiengruppe zu nennen, deren Fortentwicklung von Studie zu Studie schließlich zu den ersten Heilungen einer pädiatrischen ALL führten (Cole, 2015). Diese Therapieoptimierung ist bis heute Vorbild für viele Therapieformen der aktuellen Krebstherapie. Aber auch im Weiteren war sowohl die Behandlung der akuten Leukämie als auch der chronisch myeloischen Leukämie (CML) oft wegweisend für weitere Krankheitsentitäten. Beispielhaft seien hier die Indikationsstellung zu einer allogenen Stammzelltransplantation, die personalisierte Therapieoptimierung durch Messung der minimalen Resterkrankung (MRD), aber auch die zielgerichtete Therapie einer genetischen Alteration, des Philadelphiachromosoms t(9,22), durch ein synthetisches Medikament (Imatinib) erst in der CML, später in der Philadelphiachromosom-positiven (Ph+)-ALL, zu nennen (Ottmann *et al*, 2007). In den letzten Jahren war das erste komplette „Krebsgenom“ in der akuten myeloischen Leukämie (AML) verfügbar (Mardis *et al*, 2009), und mit einem bi-spezifischen Antikörper gegen CD3 und CD19 wurde ein neuartiges Therapieprinzip in die ALL-Therapie eingeführt (Topp *et al*, 2011). Dies sind nur einige Beispiele, die die ALL als modellhaft für Therapieweiterentwicklungen beschreibt, basierend auf der Optimierung bestehender Therapieformen, Entwicklung neuer Medikamente in Abhängigkeit zu Grunde liegender molekularer Veränderungen und besserem Verständnis der Krankheitsentstehung mittels neuer Techniken.

Vor diesem Hintergrund sei im Folgenden die T-ALL mit dem heutigen Stand der Therapie, dem molekularen Hintergrund der Erkrankung und den offenen Fragen zur Therapieresistenz, die immer noch in einer Großzahl von Patienten besteht, dargestellt.

2.2 *T-ALL als Subgruppe der ALL*

Die T-Zell akute lymphoblastische Leukämie stellt eine Unterform der ALL dar, die im Kindesalter ca. 15% aller ALL-Erkrankungen ausmacht und im Erwachsenenalter auf annähernd 25% steigt (Borowitz *et al*, 2008). Die T-ALL ist charakterisiert durch eine Proliferation von lymphoblastischen Zellen, die ihren Ursprung in frühen Formen der T-Zell-Entwicklung haben. Die Abgrenzung der T-ALL zum T-lymphoblastischen Lymphom (T-LBL), obwohl beide vermutlich von ähnlichen Vorläuferzellen ausgehend, ist anhand des Befallsmuster bei Erstdiagnose vorzunehmen. Nach der WHO-Klassifikation hat das T-LBL neben der die T-Zell-Neoplasie definierende Raumforderung im lymphatischen, thymischen oder extranodalen Gewebe keine oder eine nur geringe Beteiligung des Knochenmark-/Blutsystems. Die T-ALL ist dagegen vorwiegend über die Knochenmarksbeteiligung (Infiltration <20%) definiert, besitzt darüber hinaus jedoch auch oft eine extramedulläre Beteiligung, vor allem in der mediastinalen Thymusloge (Borowitz *et al*, 2008).

2.3 *Diagnostik der T-ALL*

Auch wenn die Verdachtsdiagnose einer ALL zunächst morphologisch aus dem peripheren Blut oder dem Knochenmark gestellt wird, ist hierüber eine Zuordnung der B-Zell- oder T-Zellreihe nicht möglich. Die morphologisch Zuordnung einer L1- (eher kleine Zellen mit hoher Kern-/Zytoplasma-Relation) oder L2- (größere Blasten mit prominenten Nukleoli) Morphologie hat neben einem deskriptiven Wert keine Bedeutung für die Diagnosestellung (You *et al*, 2015).

Entscheidenden Stellenwert in der Diagnostik besitzt die Durchflusszytometrie, die neben dem Nachweis des spezifischen zytoplasmatischen Markers cyCD3, welcher die Zugehörigkeit zur T-Zellreihe definiert, auch die Zuordnung zu therapierelevanten, immunozytologisch definierten Subgruppen ermöglicht. In der Klassifikation der „European Group for the Immunological Characterization of Leukemias“ (EGIL) werden vier Gruppen basierend auf der Expression unterschiedlicher Marker unterschieden: pro-T-ALL (cyCD3+, CD7+, CD5−, CD2−, sCD3−, CD4−, CD8−, CD1a−); prä-T-ALL (cyCD3+, CD7+, CD5+/−, CD2+/−, sCD3−, CD4−/+, CD8−/+, CD1a−); thymische T-ALL (cyCD3+, CD7+, CD5+, CD2+, sCD3+/−, CD4+/−, CD8+/−, CD1a+); und reife T-ALL (cyCD3+, CD7+, CD5+, CD2+, sCD3+, CD4+/−, CD8+/−, CD1a−) (Bene *et al*, 1995). Die beiden erstgenannten werden oft, z.B. im Kontext der „German multicenter ALL study group“ (GMALL) zur Gruppe der frühen T-ALL zusammengefasst. Darüber hinaus ist die Gruppe der ETP-ALL als Untergruppe der frühen T-ALL immunzytologisch definiert und wird noch ausführlich in dieser Arbeit beschrieben.

Zur Standarddiagnostik gehören neben der Zytogenetik zunehmend molekulargenetische Untersuchungen, auch wenn diese bislang im Gegensatz zur BCP-ALL (t(9;22); MLL-Translokationen) noch keinen Stellenwert in der Zuordnung zu therapierelevanten Gruppen der T-ALL haben. Die Klassifikation basierend auf der Durchflusszytometrie hat einen großen Stellenwert in der pragmatischen Durchführung der Diagnostik und initialen Therapiestratifizierung, allerdings trägt sie nur eingeschränkt zum Verständnis der molekularen Heterogenität der T-ALL bei. Hier scheinen regelmäßig auftretende genetische Aberrationen, welche sowohl Mutationen als auch insbesondere Translokationen umfassen, in Verbindung mit Genexpressionsprofilen eine teils überlappende, aber in entscheidenden Punkten auch abweichende Subklassifizierung nahezulegen, wie in den folgenden Abschnitten dargelegt werden soll.
Zum Abschluss der Diagnostik sei noch die obligate Liquorpunktion zum Ausschluss und gleichzeitiger prophylaktischen Therapie eines ZNS-Befalls durch die T-ALL erwähnt.

2.4 *Klinische Manifestation bei Erstdiagnose*

Klinisch manifestiert sich die Erkrankung der T-ALL oft durch Symptome, die aus der Verdrängung der gesunden Hämatopoese im Knochenmark durch die schnell proliferierende Blastenpopulation entstehen. So sind typische Erstmanifestationen Schwäche, Müdigkeit oder Leistungsknick durch die Anämie, Infekte bis hin zur Sepsis durch die Granulozytopenie oder Blutungen auf Grund der Thrombozytopenie. Neben dem Knochenmarksbefall finden sich häufig mediastinale Raumforderungen, die mit einer oberen Einflussstauung einhergehen können. Auch andere Lymphknotenstationen können betroffen sein. Darüber hinaus sind Spleno- oder Hepatomegalie häufig vorkommend. Eine seltenere, aber umso schwerere Komplikation ist ein ZNS-Befall, der in ca. 6% aller Fälle vorliegt.
Bei initial hohen Leukozytenzahlen können Symptome der Leukostase auftreten: Verwirrtheit, neurologische Ausfälle, pulmonale Verschattungen, retinale Einblutungen oder auch selten arterielle Verschlüsse.
Männer sind im Verhältnis von 3:1 häufiger als Frauen von einer T-ALL betroffen.
Ätiologisch sind keine klaren Ursachen für eine T-ALL bekannt. Risikofaktoren für eine ALL im Allgemeinen sind die Erkrankung an einer Trisomie 21 sowie Strahlenbelastung oder die Exposition gegenüber Benzol. Vorangegangene intensive Chemotherapien oder Strahlentherapien können Auslöser einer sekundären ALL sein. Diese erklären jedoch nur einen sehr kleinen Teil der Erkrankungen. Ebenso selten sind heriditäre Erkrankungen. Eine genetische Prädisposition ist Gegenstand aktueller Forschung (Perez-Andreu *et al*, 2015).

2.5 *Therapie und Prognose der T-ALL des Erwachsenen*

Erwachsene Patienten mit einer ALL im Alter bis zu 55 Jahren werden in Deutschland nahezu populationsbasiert einheitlich im Rahmen der GMALL-Studiengruppe oder analog hierzu behandelt. Hierbei werden BCP- und T-ALL im Grundsatz einheitlich behandelt, lediglich die reife B-ALL wird hiervon abweichend behandelt. Zurzeit ist das GMALL-Protokoll 08/2013 in Vorbereitung. Das über die Jahre hinweg kontinuierlich weiter entwickelte Protokoll besteht aus drei wesentlichen Therapiephasen, die ausgehend von Protokollen aus pädiatrischen Studiengruppen (s. oben, BFM-Studiengruppe und St.-Jude-Krankenhaus) oft als pädiatrisch basierte ALL-Therapien bezeichnet werden.

Im Rahmen einer Induktionstherapie ist das Erreichen einer kompletten Remission das Ziel, welches in über 80% aller Patienten erreicht werden kann (Hoelzer *et al*, 2016). Hierbei kommen in der Vorphase Dexamethason und Cyclophophamid zum Einsatz, anschließend Anthrazykline, Vincristin, Cytarabin, Mercaptopurin und als „ALL-spezifische“ Therapie PEG-Asparaginase.

Im Rahmen der Konsolidierung kommt der Risikostratifizierung eine entscheidende Bedeutung zu. Aktuell werden in der T-ALL die immunzytologisch definierten Subgruppen der early T-ALL und reifen T-ALL als Hochrisiko betrachtet. Zusätzlich kommt der MRD-Messung eine zentrale Rolle zu, so dass Patienten mit einem Therapieversagen (MRD > 10^{-4} an Tag 71 und Woche 16) ebenfalls der Hochrisikogruppe zugeordnet werden. Für diese Patienten wird bei vorhandenem Fremdspender eine rasche allogene Stammzelltransplantation in erster kompletter Remission angestrebt. Für alle übrigen Patienten besteht die Konditionierung aus einer Abfolge wechselnder Polychemotherapien, die auch einen Re-Induktionsblock umfassen. Hierbei kommen Vindesin, Etoposid, Hochdosis Methotrexat, Hochdosis Cytarabin und Medikamente der Induktionstherapie zum Einsatz.

Als dritte Therapiephase scheint eine Erhaltungstherapie mit Mercaptopurin und Methotrexat für Patienten, die keine allogene Stammzelltransplantation erhalten, unentbehrlich. Eventuell hat PEG-Asparaginase auch in der Erhaltungstherapie einen Stellenwert. Insgesamt dauert die gesamte Therapie einschließlich der Erhaltungstherapie zwei bis zweieinhalb Jahre.

Zusätzliche Elemente der Therapie in der BCP-ALL sind bei CD20-Positivität der Anti-CD20-Antikörper Rituximab und für Ph+-Patienten ein TKI, standardmäßig Imatinib. Für die T-ALL besteht bislang keine derartige zielgerichtete Therapieergänzung.

Prophylaktisch sind in die Therapie eine intrathekale ZNS-Therapie mit Methotrexat, Dexamethason und Cytarabin sowie eine Bestrahlung des ZNS und gegebenenfalls des Mediastinums integriert.

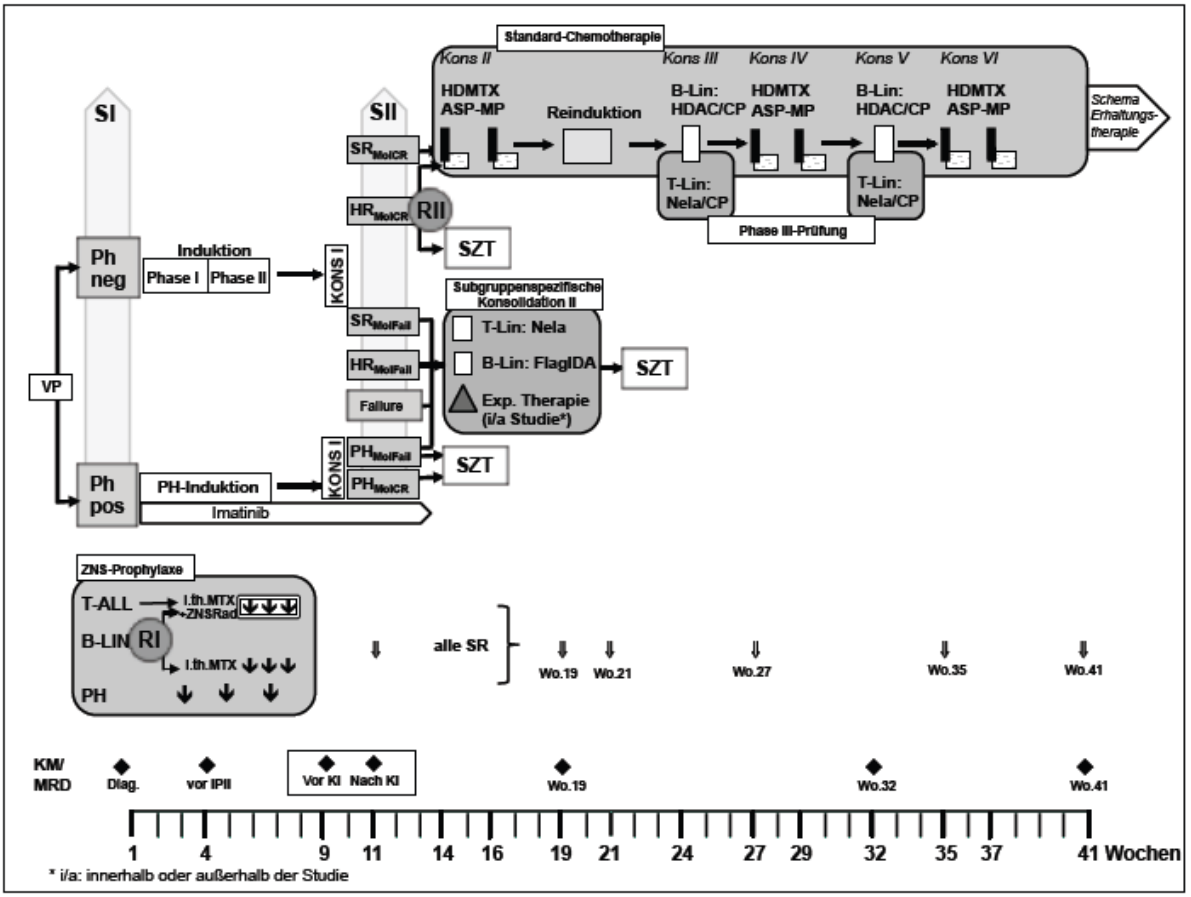

Abbildung 1. Therapieschemata der GMALL-Studie 08/2013. Das Schema stellt den Studienablauf der aktuellen GMALL-Studie dar, welches die wesentliche Elemente der vorangegangenen Studienprotokolle übernimmt, ergänzt im Wesentlichen um eine weitere Intensivierung der MRD-Stratifizierung und dem Einschluss von experimentellen Therapien im Rezidiv oder bei unzureichender MRD-Tiefe.

Mit dem dargestellten Therapieschema (Abb. 1) beträgt das 5-Jahresüberleben in der Gruppe der Hochrisikopatienten aktuell im Rahmen der GMALL 50%, in der Gruppe der Patienten mit Standradrisiko 64% (GMALL-Studienprotokoll 08/2013).

In der Rezidivsituation sind die Therapieaussichten reduzierter und die Therapiekonzepte nicht standardisiert. Im Allgemeinen wird eine allogene Stammzelltransplantation angestrebt, bei bereits erfolgter allogener Stammzelltransplantation auch eine zweite. Voraussetzung hierfür ist das Erzielen einer zweiten kompletten Remission durch eine Rezidivtherapie. Hierbei kommen oft Protokolle mit Anthrazyklinen, Fludarabin und Hochdosis Cytarabin zum Einsatz (z.B. Ida-FLAG oder Mito-FLAG) (Gökbuget *et al*, 2012).

2.6 *Unterschiede der pädiatrischen und erwachsenen T-ALL*

Angesichts der gravierenden Unterschiede in der Prognose für pädiatrische und erwachsene Patienten mit einer Rezidivwahrscheinlichkeit von 15% versus 50%, stellt sich die Frage nach den Ursachen hierfür und nach möglichen Therapieoptimierungen. Wie schon angesprochen, basiert auch das oben angesprochene Therapieprotokoll auf Elementen, die so auch bei Kindern verwendet werden. Und tatsächlich scheint sich das Überleben für adoleszente Patienten im Alter von 16-21 Jahren, die uneinheitlich mal im pädiatrischen, mal im erwachsenen Kontext behandelt werden, nicht signifikant in Abhängigkeit des Protokolls zu unterscheiden (Usvasalo *et al*, 2008). Das Gesamtüberleben verschlechtert sich im Rahmen der GMALL-Studie in Einklang mit anderen internationalen Studiengruppen mit fortschreitendem Alter kontinuierlich. Während das 5-Jahres-Überleben für 16-25-Jährige noch 70% beträgt, sinkt es für 36-45-Jährige auf 52% und für 56-65-Jährige auf 34%.

Ein Teil dieser Prognoseverschlechterung ist sicherlich durch zunehmende Komorbiditäten und schlechterer Therapieadhärenz zu erklären, aber auch für Patienten ohne erkennbare Komorbiditäten ist bei kompletter Durchführung eine erhöhte Therapierefraktarität im Alter festzustellen. Hierfür sind zumindest zum Teil molekulare Unterschiede verantwortlich. Als prominentes Beispiel ist die bcr/abl-Translokation zu nennen, die mit einer schlechten Prognose assoziiert ist und im Alter eine zunehmende Inzidenz zeigt (Herold *et al*, 2014). Inwieweit weitere molekulargenetische Charakteristika die ungünstige Prognose im Alter begünstigen und gegebenenfalls durch gezielte Therapien angegangen werden könnten, ist Gegenstand aktueller Untersuchungen. So scheint z.B. eine komplett chemofreie Therapie einer *BCR/ABL*-positiven BCP-ALL im älteren Patienten mit einem Tyrosinkinaseinhibitor und dem bispezifischen CD19/CD3-Antikörper Blinatumomab eine gangbare Alternative zu sein.

Für die T-ALL sind altersspezifische molekulare Veränderungen bislang noch nicht bekannt.

3. HOCHDURCHSATZVERFAHREN ZUR CHARAKTERISIERUNG DER T-ALL

Bei hämatologischen Erkrankungen insgesamt und hier in der T-ALL im Speziellen hat die Etablierung von Hochdurchsatzverfahren zur Sequenzierung zu einem Anstieg der molekularen Daten geführt. Im Einzelnen seien hieraus Erkenntnisse bzgl. genspezifscher Alterationen, Expressionsänderungen und Detektierung von Translokationen in der T-ALL dargestellt.

3.1 *Rekurrent mutierte Gene in der T-ALL*

Während bestimmte genetische Alterationen in der T-ALL (u.a. *NOTCH1*, *FBXW7*, *WT1*, *CDKN2A/B*) bereits lange bekannt sind, hat sich das Bild durch das Aufkommen von Hochdurchsatzverfahren deutlich erweitert. Es sind in der T-ALL Untersuchungen mittels „whole genome sequencing“ (WGS), „whole exome sequencing“ (WES), „RNA sequencing“ (RNAseq) und auch mittels Genpanel zur Charakterisierung der T-ALL auf Ebene der einzelnen Base durchgeführt worden (u.a. Zhang *et al*, 2013; Atak *et al*, 2013; Neumann *et al*, 2015; De Keersmaecker *et al*, 2013). Hierbei zeigte sich ein deutlich heterogeneres Bild als erwartet. Neben den in über 50% aller Patienten alterierten Genen *NOTCH1* und *CDKN2A/B*, für die bislang trotz zahlreicher früher Studien bislang keine therapeutische Konsequenz abgeleitet werden konnte, war kein weiteres Gen in einem Prozentsatz von über 20% alteriert. Allerdings konnten für mehrere Gene Mutationsfrequenzen im Bereich von 5-15% bestätigt oder neu bestimmt werden: *FBXW7* (Baldus *et al*, 2009), *WT1 (*Heesch *et al,* 2010), *PHF6* (Van Vlierberghe *et al*, 2010), *MEF2C* (Zuurbier *et al*, 2014), *BCL11B* (Gutierrez *et al*, 2011), *RUNX1* (Grossmann *et al*, 2013), *NRAS* (Neumann *et al*, 2015), *JAK1* (Flex *et al*, 2008), *JAK3* (Zhang *et al*, 2012) und weitere. Für eine zielgerichtete Therapie erwächst aus dieser Heterogenität die Herausforderung einer hoch personalisierten Diagnostik und Therapie. Insgesamt sind in der Mehrzahl der Patienten (>80%) Signalwege affektiert, für die eine hypothetische zielgerichtete Therapieoption besteht (Neumann *et al*, 2015). Neben dem bereits oben besprochenen NOTCH-Signalweg ist hier vor allem der JAK/STAT-, der PI3K-Akt-mTOR- und der WNT-Signalweg zu nennen. Darüber hinaus eröffnen regelmäßig auftretende Veränderungen in epigenetischen Modulatoren (*EZH2*, *SUZ12*, *EED*, *MLL2*, *DNMT3A;* Zhang *et al*, 2012; Neumann *et al*, 2013c) interessante therapeutische Optionen, die für einen zielgerichteten Einsatz allerdings weiterer funktioneller Charakterisierung bedürfen. Zuletzt seien Mutationen erwähnt, die zwar bei Erstdiagnose selten auftreten, aber im Rezidiv wiederkehrend zu finden sind. *NT5C2* konnte in 20% der rezidivierten

pädiatrischen T-ALL nachgewiesen werden (Meyer *et al*, 2013; Tzoneva *et al*, 2013). Die Therapierefraktärität wird vermutlich über eine Resistenz gegen 6-Mercaptopurin und 6-Thioguanin vermittelt, welche elementare Bestandteile der Standardchemotherapie sind. Während die meisten Untersuchungen an pädiatrischen Patienten durchgeführt worden sind, ist das Wissen bei erwachsenen Patienten limitierter. Es gibt Hinweise darauf, dass sich die Mutationsprofile deutlich voneinander unterscheiden. Exemplarisch seien hierfür die rekurrenten Mutationen von *DNMT3A* und *MLL2* im Erwachsenen genannt, welche bei pädiatrischen Patienten nicht gefunden worden sind (Neumann *et al*, 2013c). Dies passt in das Bild einer alternden Hämatopoese mit potentiell vorliegenden präleukämischen Mutationen auf Stammzellniveau (Genovese *et al*, 2014; Jaiswal *et al*, 2014). Entsprechend finden sich Mutationen von *DNMT3A* vorwiegend bei älteren Patienten mit unreifen T-ALL-Formen.

3.2 *Genexpressionsprofile definieren Subgruppen der T-ALL*

Untersuchungen von Genexpressionsprofilen in der T-ALL haben gezeigt, dass es wenige Subgruppen (4-5) gibt, die dem Genexpressionsmuster von verschiedenen Stadien der physiologischen T-Zell-Entwicklung entsprechen (Ferrando *et al*, 2002). Diese Gruppen unterscheiden sich in der Überexpression verschiedener Onkogene und korrelieren sowohl mit dem Immunphänotyp als auch mit zu Grunde liegenden genetischen Alterationen (Abb. 2; Meijerink *et al*, 2010). So ist die Subgruppe mit Aktivierung von *TLX1* eng mit der Expression von CD1 (thymische Subgruppe) verknüpft, während Patienten mit *TAL1/LMO*-Aktivierung eher den Immunphänotyp einer reifen T-ALL aufweisen. In einem Großteil der T-ALL-Patienten mit aberranter Expression von HOXA-Genen lässt sich ein Fusionsgen von *MLL* oder *AF10* mit verschiedenen Partnergenen nachweisen (Dik *et al*, 2005; Ferrando *et al*, 2004). Ein DOTL1-Inhibitor, der sich in früher klinischer Testung befindet, könnte hier eine zusätzliche Therapieoption darstellen (Daigle *et al*, 2013).

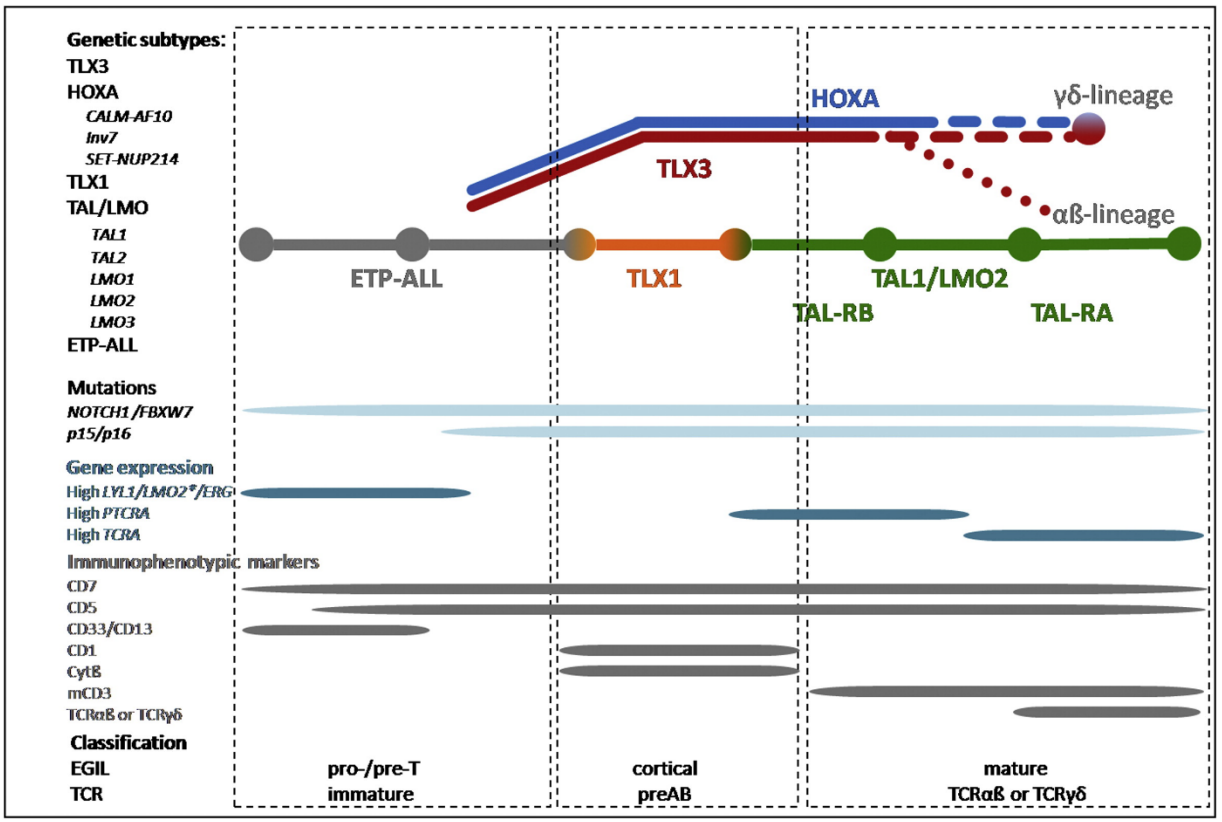

Abbildung 2: **Korrelation von Genexpression zu Immunphänotyp und genetischen Alterationenen** Schematische Darstellung der genetischen Subgruppen der T-ALL (TLX3, TLX1, HOXA und TAL/LMO) in Relation zu dem Differenzierungsgrad der T-Zell Entwicklung. Der Nachweis von spezifischen Mutationen (p15/p16 Deletionen, NOTCH1) und die Expression von Kandidatengenen (LYL1, LMO2, ERG, PTCRA, TCRA) sind dargestellt (Meijerink *et al*, 2010).

Besondere Aufmerksamkeit auf Grund ihrer distinkten Charakteristika hat in den letzten Jahren die Subgruppe der ETP-ALL gewonnen. Diese wurde basierend auf einem Genexpressionsprofil, welches physiologischen frühen tyhmischen Progenitoren („early thymic progenitors“) entspricht, identifiziert (Coustan-Smith *et al*, 2009; Abb. 3). Sie tritt bei ca.10% aller T-ALL Patienten auf und weist eine vermeintlich ungünstige Prognose auf. Dies führte in der pädiatrischen ETP-ALL zur Empfehlung einer frühen allogenen Stammzelltransplantation. Weiterhin weist die ETP-ALL hinsichtlich Genexpression, Immunphänotyp und Mutationsprofil Merkmale einer Stammzellleukämie auf (Neumann *et al,* 2012; Zhang *et al*, 2012; Abb.3). Insbesondere das gehäufte Vorkommen von Mutationen im JAK/STAT-Pathway, epigenetischen Regulatoren und das Auftreten von *FLT3*-Mutationen, bieten alternative Therapieansätze (Zhang *et al*, 2012; Neumann *et al*, 2013a). So wurde im Tierversuch bereits der JAK/STAT-Inhibitor Ruxolitinib als effektive Therapieoption evaluiert (Maude *et al*, 2015).

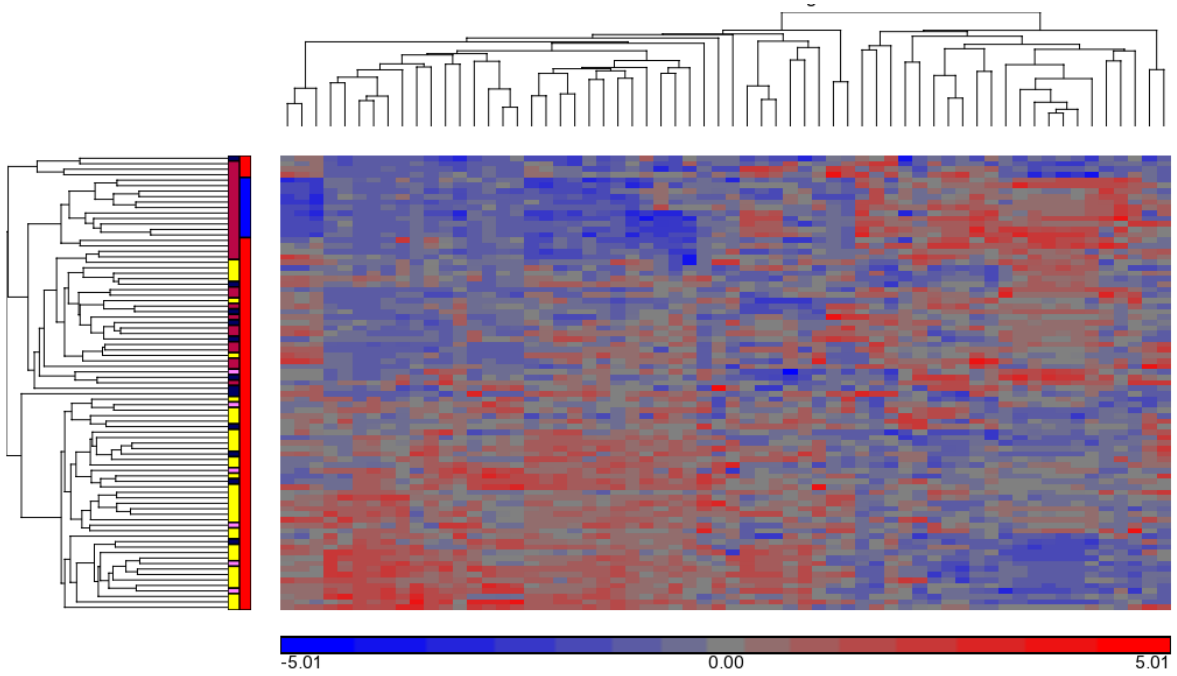

Abbildung 3: **ETP-ALL in der erwachsenen T-ALL**, **Genexpressionsprofile von 83 T-ALL-Patienten** Genexpressionsprofile (Affymetrix HG-U133plus2) von 83 T-ALL-Patienten der GMALL-Studiengruppe. „Unsupervised hierarchial clustering“ der Gensignatur von Coustan-Smith (55 „probe sets“; Couston-Smith *et al*, 2009) zeigt ein distinktes Cluster mit der Expression einer ETP-ALL (ETP-ALL blau; non-ETP-ALL rot; AG Baldus, unpublizierte Daten).

Allgemein ist zu ergänzen, dass die Charakterisierung von Subgruppen an Hand von Genexpressionsprofilen zu Therapieänderungen nicht nur für die ETP-ALL gezeigt worden ist. Nachdem einzelne prognostische Marker bezüglich ihrer Expression oft keinen Eingang in den klinischen Alltag gefunden haben, ist es mit dem Einzug der neuen Hochdurchsatzverfahren möglich, neben einer prognostischen Einschätzung auch bereits therapeutische Zielstrukturen zu identifizieren. Beispielhaft ist dies für den Einsatz von zielgerichteten Therapien entsprechend der individuellen genetischen Alterationen für einzelne Patienten der an Hand des Genexpressionsprofil identifizierten Ph-like ALL Subgruppe gezeigt worden (Mullighan *et al*., NEJM 2009; Roberts *et al*., NEJM 2014; Weston *et al*., JCO, 2013). Durch den Einsatz von Hochdurchsatzverfahren konnte eine Vielzahl von verschiedenen, neuen molekularen Veränderungen bei der ALL identifiziert werden. Aus diesen müssen diagnostisch und therapeutisch verwertbare Zielstrukturen durch die Zusammenarbeit von technisch-methodisch, biologisch und klinisch orientierten Forschergruppen herausgefiltert und validiert werden.

3.3 *Fusionsgene als onkogene Alterationen in der T-ALL*

In der Leukämogenese der T-ALL spielen Translokationen, die zur aberranten Expression von Onkogenen führen, eine charakteristische Rolle und sind in einer Vielzahl von T-ALL-Patienten zu finden (van Vlierberghe *et al*, 2013). Auch wenn viele chromosomale Bruchpunkte bereits in der konventionellen Zytogenetik zu detektieren sind, hat erst die Sequenzierung der in der Zelle vorhandenen RNA (RNAseq) oder des gesamten Genoms (WGS) zur umfassenden Charakterisierung von auch seltenen, teils kryptisch vorliegenden Fusionsgenen geführt.

Ein typisches Motiv dieser Fusionsgene ist die Translokation von T-Zell spezifischen Transkriptionsfaktoren in Regionen der T-Zell-Antigen-Rezeptoren. Die so erzielte Deregulation dieser Transkriptionsfaktoren führt zur onkogenen Aktivierung. Als prominentestes Beispiel sei hier *TAL1* als Mitglied der bHLH-Familie genannt. Für *TAL1* sind jeweils in ca. 3% aller pädiatrischen T-ALL-Patienten die Translokationen t(1;14)(p32;q11) oder t(1;7)(p32;q34) beschrieben, die den Transkriptionsfaktor unter die Kontrolle der TCRA/D-Enhancer stellt (Aplan *et* al, 1990). Deutlich häufiger ist jedoch eine kleine intrachromosomale Deletion im Chromosom 1, die zur aberranten Expression von *TAL1* führt, indem *TAL1* unter die Kontrolle des hoch exprimierten Gens *STIL* gestellt wird (Bash *et* al, 1995). Neben *TAL1* sind auch *TAL2* oder *LYL1* als weitere bHLH-Familienmitglieder regelmäßig durch Translokationen aktiviert. Des Weiteren sind *TLX1*, *TLX3*, *HOXA* (direkt und indirekt) sowie *NKX2*.1 und *NKX2*.2 als Homeoboxgene und *LMO1*, *LMO2*, und *LMO3* als Mitglieder der LMO-Familie durch Fusionen aberrant gesteigert (van Vlierberghe *et al*, 2012).

3.4 *Epigenetische Muster der T-ALL*

Ausgehend von der ursprünglichen Begriffsdefinition von epigenetischen Prozessen als generationsübergreifend vererbten Prozessen, die unabhängig von der genetischen Kodierung sind und im Wesentlichen DNA-Methylierungen betreffen (Waddington, 2012), werden unter epigenetischen Phänomenen in einer weiter gefassten Definition auch generell chromatinmodifizierende Prozesse subsummiert (Berger *et al*, 2009). Sowohl DNA-Methylierungen als auch Histonmodifikationen sind eng mit dem transkriptionellen Status des Genoms verknüpft. Daher ist es nicht verwunderlich, dass epigenetische Alterationen und das aktive Wechselspiel zwischen DNA-Methylierung und Histonmodifikationen zentraler Bestandteil der Tumorbiologie sind und zu einer Zelltransformation führen können (Hanahan *et al*, 2011).

In der T-ALL sind zahlreiche genetische Alterationen von epigenetischen Modulatoren beschrieben. Die Auswirkungen der genetischen Alterationen in epigenetischen Modulatoren sind in der T-ALL allerdings noch unzureichend untersucht. In der AML sind Mutationen in Genen (*DNMT3A*, *TET2*; Ley *et al*, 2010; Delhommeau *et al*, 2009), die in DNA-Methylierung involviert sind, nahezu ausschließlich im Hauptklon zu finden. Des Weiteren sind sie sowohl in gesunder alternder Hämatopoese als mögliche präleukämische Läsion (Genovese *et al*, 2014; Jaiswal *et al*, 2014) als auch in kompletter Remission nach erfolgter Chemotherapie zu finden (Debarri *et al*, 2015). Dies spricht dafür, dass es sich um erste, frühe genetische Alterationen handelt. In der AML ist ein distinktes globales Methylierungsmuster beschrieben, welches insbesondere mit *DNMT3A*R882-Mutationen einhergeht und prognostische Signifikanz besitzt (Qu *et al*, 2014; Ribeiro *et al*, 2012). In der T-ALL existieren nur limitierte Untersuchungen zu genomweiten Methylierungsmustern, die sich auf pädiatrische Patienten beschränken (Nordlund *et al*, 2013; Borssén *et al*, 2013). Diese werden sich auch auf Grund der fehlenden *DNMT3A*-Mutationen deutlich von erwachsenen Patienten unterscheiden. In pädiatrischen Patienten scheint eine global niedrige DNA-Methylierung mit schlechtem Therapieansprechen und einem unreifen Phänotyp assoziiert zu sein (Borssén *et al*, 2013; Nordlund *et al*, 2015). Auch die ETP-ALL scheint ein distinktes Methylierungsprofil aufzuweisen, welches Ähnlichkeiten zur AML besitzt (eigene unveröffentlichte Daten, Abb. 4).

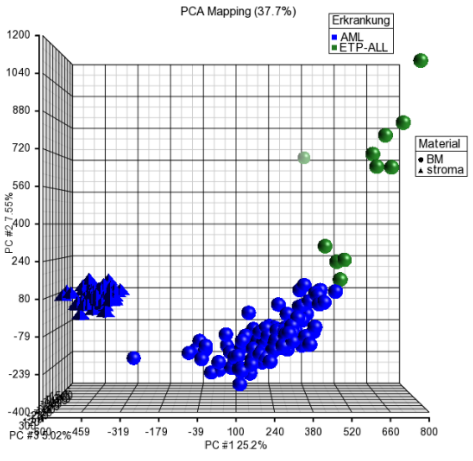

Abbildung 4. Globale Methylierungsprofile für Patienten mit AML oder ETP-ALL

Principal component analysis für globale Methylierungsmuster (Illumina bead chip array 450k) von AML-Patienten (KM: blaue Kugeln; Stroma: blaue Pyramiden). Von den zwölf ETP-ALL-Proben (KM: grüne Kugeln) zeigen acht Proben ein sehr distinktes Methylierungsprofil, vier ähneln dem Methylierungsprofil von AML-Patienten (AG Baldus, unveröffentlichte Daten).

Neben der veränderten DNA-Methylierung ist vor allem die Affektion von Genen, die in posttranslationale Histonmodifikationen involviert sind, auffällig. Insbesondere Gene des „polycomb repressor complex 2“ (PRC2) weisen eine hohe Rate an Mutationen auf (Zhang *et al*, 2012). In einem Mausmodell führte der knock-out von *EZH2*, einem Gen des

PRC2, in der Hämatopoese zur Entwicklung eines T-Zell-Tumors (Simon *et al*, 2012). Ferner konnte in Xenograft-Modellen von T-Zell-Linien für PRC2-Gene Tumorsuppressorcharakteristika gezeigt werden (Nztiachristos *et al*, 2012). Und zuletzt kooperiert eine verminderte Methylierung von H3K27, vermittelt durch PRC2, mit der Aktivierung von *NOTCH1* während der T-ALL-Entwicklung (Nztiachristos *et al*, 2012).
Zusammengenommen scheint eine präexistierende Alteration des PRC2 mit weiteren aktivierenden Läsionen in der Pathogenese einiger T-ALL-Patienten eine entscheidende Rolle zu spielen. Herausfordernd bleibt, inwieweit sich diese Veränderungen therapeutisch modulieren lassen. Obwohl Therapieoptionen in frühen Studien (präklinisch, Phase I) bestehen (DOT1L-Inhibitoren, HDAC-Inhibitoren, DNMT-Inhibitoren, BRD4-Inhibitoren und weitere), ist unklar, welche T-ALL-Patienten tatsächlich hiervon profitieren könnten. Informationen über genomweite Methylierungsmuster, Histonomodifikationen, aber auch Sensitivität von Xenograft-Modellen auf epigenetische Therapie in Abhängigkeit ihres Mutationsprofils fehlen bislang für erwachsene T-ALL-Patienten.

4. THERAPEUTISCHE STRATEGIEN

Die Therapieoptimierung in der akuten Leukämie erfordert neben der umfassenden klinischen Charakterisierung auch die Erfassung von molekularen Veränderungen zur Prognoseabschätzung und Therapieanpassung. Die GMALL ist eine der weltweit größten Studiengruppen zur Therapieoptimierung der ALL und ermöglicht durch den Einschluss zahlreicher Kliniken und die Vereinheitlichung der Therapie eine populationsrepräsentative Beobachtung des Verlaufs der Erkrankung. Dies ermöglicht auf Grund der vorliegenden umfangreichen Biobanken eine einmalige Möglichkeit des Brückenschlags von Untersuchungen molekularer Marker hin zu klinisch relevanten Parametern.
Aktuell wird in der T-ALL eine Risikoeinteilung bei Diagnose auf Grund des Immunphänotyps vorgenommen (Gökbuget *et al*, 2012). Hierbei werden die Gruppen der frühen und reifen T-ALL als Hochrisiko eingestuft und bei vorliegendem Spender eine allogene Stammzelltransplantation in erster kompletter Remission empfohlen. Zusätzlich ist, basierend auf dem MRD-Niveau, eine Therapieintensivierung mittels allogener Stammzelltransplantation bei molekular persistierender Erkrankung vorgesehen (Brüggemann *et al*, 2012). Im Rezidiv wird ebenfalls eine allogene Stammzelltransplantation, ggfs. auch eine zweite, angestrebt (Gökbuget *et al*, 2012). Zielgerichtete Therapien, wie sie in der B-Vorläufer(BCP)-ALL etabliert sind (z.B.

Rituximab (Thomas *et al*, 2010), Blinatumomab (Topp *et al*, 2012), Imatinib (Ottmann *et al*, 2007)), fehlen in der Behandlung der T-ALL. *NOTCH1*, in 50-70% der T-ALL-Patienten aktivierend mutiert (Baldus *et al*, 2009; Ferrando *et al*, 2010), stellt das naheliegendste Ziel dar, aber frühe Studien mit γ-Sekretase-Inhibitoren oder Antikörpern verliefen unbefriedigend und fanden bislang noch keinen Eingang in therapeutische Standardregime (Litzow *et al*, 2015).

Daher sind zwei Strategien notwendig, die verfolgt werden müssen: zum einen müssen Patienten mit hohem Rezidivrisiko frühzeitig identifiziert werden und ggfs. einer Therapieintensivierung zugeführt werden. Zum anderen sollten molekulare Charakteristika möglichst frühzeitig erfasst werden, die in einer MRD-positiven Situation eine frühe zielgerichtete Therapie durch entweder schon in anderen Krankheitsentitäten etablierte oder noch zu entwickelnde Medikamente erlauben.

5. FRAGESTELLUNG UND ZIELSETZUNG

Im Mittelpunkt der hier vorgestellten eigenen Arbeiten steht die umfassende molekulare Charakterisierung der T-ALL im Erwachsenenalter zur Therapieoptimierung. Dies ist insbesondere von Interesse, da es für die T-ALL im Gegensatz zur B-Vorläufer-ALL noch keine etablierten spezifischen zielgerichteten Therapien gibt. Die in den letzten Jahren verbesserte Prognose ist vorwiegend auf eine optimierte Risikostratifizierung, unter anderem durch die Messung des MRD-Niveaus, mit Therapieintensivierung durch eine allogene Stammzelltransplantation zurückzuführen.

TEIL I

Hieran anknüpfend wird im ersten Teil nachgegangen, welche Rolle die Subgruppe der ETP-ALL im Kontext der erwachsenen T-ALL spielt. Dies kann basierend auf der Immunzytologie, Standard der initialen Diagnostik bei jedem T-ALL-Patienten, identifiziert werden. Die vorgenommene molekulare Charakterisierung in Korrelation zu klinischen Überlebensdaten zeigen eine Stammzellleukämie mit myeloischen Eigenschaften, die alternative Therapieschemata, angelehnt an die Behandlung einer AML, als Option erscheinen lassen. Dies wird insbesondere für die in dieser Subgruppe gehäuft und für die T-ALL nahezu exklusiv auftretenden *FLT3*-Mutationen näher untersucht.

TEIL II

Im zweiten Teil der Arbeit werden die Gene *BCL11B*, ein wichtiger Transkriptionsfaktor der T-Zell-Entwicklung, und *FAT1*, ein Cadherin mit Implikationen über den WNT-Signalweg in vielen Tumorformen, untersucht. Expression und Mutationen werden charakterisiert und auf ihre prognostische Wertigkeit untersucht. Insbesondere die Identifizierung von distinkter Expression in der Gruppe der thymischen T-ALL machen diese Gene zu interessanten Kandidaten für eine frühzeitige Identifizierung von T-ALL-Patienten mit ungenügendem Ansprechen auf die Standardtherapie.

TEIL III

Schließlich werden im dritten Teil der Arbeit Kollektive von T-ALL-Patienten umfassend unter Einbeziehung von Hochdurchsatztechnologien charakterisiert. Im Stammzellleukämie-Modell der ETP-ALL werden mittels gesamter Exomsequenzierung neue rekurrente Mutationen detektiert, die in epigenetische Regulationsmechanismen eingebunden sind. Mittels Genpaneluntersuchungen wurden basierend auf diesem

Kollektiv 83 T-ALL Patienten auf ihren Mutationsstatus von 88 Genen untersucht. Hier zeigten sich in einem Großteil der Patienten Alterationen in Signalwegen, die theoretisch einer therapeutischen Intervention zugänglich wären. Besonders hervorzuheben sind hier epigenetische Alterationen, die zuvor nur einem kleinen Prozentsatz der T-ALL-Patienten beschrieben waren.

6. EIGENE ARBEITEN

Im Folgenden werden die Strategien zur Therapieoptimierung der erwachsenen T-ALL basierend auf einer subgruppenspezifischen Risikostratifizierung, Charakterisierung prognostischer Marker und Identifizierung molekularer Zielstrukturen an den eigenen Arbeiten dargestellt.

6.1 *TEIL I: Subgruppenbasierte Therapieoptimierung am Beispiel der ETP-ALL*

In der T-ALL des Erwachsenen hat die Unterscheidung in Subgruppen entsprechend ihrem Immunphänotyp und daraufhin erfolgende Therapieintensivierung zu einem verbessertem Überleben geführt. Allerdings ist auch innerhalb der Subgruppen noch kein optimales Therapieregime gefunden worden und insbesondere innerhalb der Hochrisikogruppen der reifen und frühen T-ALL das Ergebnis noch unbefriedigend.

6.1.1 ***Klinische und molekulare Charakterisierung der ETP-ALL im Erwachsenenalter***

(**Neumann M**, Heesch S, Gökbuget N, Schwartz S, Schlee C, Benlasfer O, Farhadi-Sartangi N, Thibaut J, Burmeister T, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. Clinical and molecular characterization of early T-cell precursor leukemia: a high-risk subgroup in adult T-ALL with a high frequency of *FLT3* mutations. Blood Cancer J. 2012;2:e55.)

In dieser Arbeit wurde die prognostische Bedeutung der ETP-ALL bei Erwachsenen untersucht. Die ETP-ALL ist gekennzeichnet durch ein Genexpressionsprofil, welches dem physiologischen Transkriptionsprogramm von frühen thymischen T-Zell-Progenitoren ähnelt. Diese Subgruppe der T-ALL ist durch einen spezifischen Immunphänotyp charakterisiert ($CD7^+$, $CD5^{weak}$, $CD1^-$, $CD8^-$, Koexpression von Stammzell- (CD34, CD117, HLA-DR) oder myeloischen (CD33, CD13, CD14) Markern). Bei pädiatrischen Patienten ist diese Subgruppe mit einem äußerst ungünstigen Risikoprofil verbunden. Wir charakterisierten die ETP-ALL bei erwachsenen T-ALL-Patienten. Hier bilden die Patienten mit ETP-ALL eine Untergruppe der early T-ALL. Von 178 Patienten mit einer early-T-ALL zeigten 57 Patienten den Immunphänotyp einer ETP-ALL, dies entspricht 32% aller early T-ALL-Patienten oder 7.5% aller erwachsenen T-ALL-Patienten. Während klinische Parameter wie Alter, Geschlecht, Leukozytenzahl bei Diagnose oder ZNS-Befall keinen Unterschied aufwiesen, zeigten ETP-ALL-Patienten signifikant seltener einen mediastinalen Befall (28% vs, 47%, p=0.02). Das 10-Jahresüberleben für Patienten mit einer ETP-ALL betrug 35%. Dies liegt in der Größenordnung der Patienten mit dem prognostisch ungünstigem Immunphänotyp einer early T-ALL (37%). Damit ist auch für

ETP-ALL-Patienten die allogene Stammzelltransplantation in erster kompletter Remission die empfohlene Therapie. Auf molekularer Ebene zeigten ETP-ALL-Patienten eine gesteigerte Expression von Genen, die auf hämatopoetischen Stammzellen zu finden sind (*BAALC*, *MN1*, *WT1* und *IGFBP7*). Während *NOTCH1*- (~60%) und *FBXW7*-(~10%) Mutationen typisch für die T-ALL sind, finden sich diese nur in einem geringen Prozentsatz in der hier untersuchten ETP-ALL-Kohorte (*NOTCH1* 8%, *FBXW7* 0%). Bemerkenswerterweise fanden wir *FLT3*-Mutationen in sieben der 129 untersuchten T-ALL-Patienten, alle in der Gruppe der early T-ALL, und sechs der sieben Mutationen betrafen Patienten mit einer ETP-ALL. Insgesamt zeigen die molekularen Analysen Hinweise auf das Vorliegen einer Stammzellleukämie und bilden konsekutiv die Rationale für zielgerichtete Therapiemöglichkeiten wie z.B. Tyrosinkinaseinhibitoren oder AML-orientierte Therapieschemata.

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ORIGINAL ARTICLE

Clinical and molecular characterization of early T-cell precursor leukemia: a high-risk subgroup in adult T-ALL with a high frequency of FLT3 mutations

M Neumann[1], S Heesch[1], N Gökbuget[2], S Schwartz[1], C Schlee[1], O Benlasfer[1], N Farhadi-Sartangi[1], J Thibaut[1], T Burmeister[1], D Hoelzer[2], W-K Hofmann[3], E Thiel[1] and CD Baldus[1]

A subgroup of pediatric acute T-lymphoblastic leukemia (T-ALL) was characterized by a gene expression profile comparable to that of early T-cell precursors (ETPs) with a highly unfavorable outcome. We have investigated clinical and molecular characteristics of the ETP-ALL subgroup in adult T-ALL. As ETP-ALL represents a subgroup of early T-ALL we particularly focused on this cohort and identified 178 adult patients enrolled in the German Acute Lymphoblastic Leukemia Multicenter studies (05/93–07/03). Of these, 32% (57/178) were classified as ETP-ALL based on their characteristic immunophenotype. The outcome of adults with ETP-ALL was poor with an overall survival of only 35% at 10 years, comparable to the inferior outcome of early T-ALL with 38%. The molecular characterization of adult ETP-ALL revealed distinct alterations with overexpression of stem cell-related genes (*BAALC*, *IGFBP7, MN1, WT1*). Interestingly, we found a low rate of *NOTCH1* mutations and no *FBXW7* mutations in adult ETP-ALL. In contrast, *FLT3* mutations, rare in the overall cohort of T-ALL, were very frequent and nearly exclusively found in ETP-ALL characterized by a specific immunophenotype. These molecular characteristics provide biologic insights and implications with respect to innovative treatment strategies (for example, tyrosine kinase inhibitors) for this high-risk subgroup of adult ETP-ALL.

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Keywords: acute lymphoblastic leukemia; ETP-ALL; T-ALL; molecular characteristics; FLT3; NOTCH1

INTRODUCTION

Early T-cell precursors (ETPs) are intrathymic c-Kit^{hi} double-negative (DN)1 cells, which contribute to the development of T-lymphocytes.[1,2] These cells represent immature progenitors that have recently immigrated from the bone marrow to the thymus. In addition to their T-lymphoid potential, they carry a natural killer, dendritic, and remarkably, myeloid cell differentiation potential.[3]

Recently, a small subgroup of pediatric acute T-lymphoblastic leukemia (T-ALL) was described showing a gene expression profile closely linked to the expression signature of ETPs.[4,5] This subtype, termed ETP-ALL, is characterized by a specific immunophenotype: CD1a^{-}, CD8^{-}, CD5^{weak} and expression of stem cell or myeloid markers. Moreover, ETP-ALLs are distinguished by high expression of oncogenic transcription factors, including genes involved in the pathogenesis of T-ALL like *LMO1*, *LYL1* and *ERG*. Pediatric patients with ETP-ALL showed a highly unfavorable outcome compared with typical T-ALL.[4] On the basis of these results, the Italian national study Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) and St-Jude Children's hospital proposed modifications in their treatment schedules for children with ETP-ALL towards a more intensive regime including allogeneic stem cell transplantation (alloSCT).[4] Up to date, the existence of a subtype with an ETP gene expression signature in adult patients has not yet been investigated.

Although several molecular risk factors with prognostic and potential therapeutic implications have been identified, such as mutations (*NOTCH1*) or aberrant expression (*BAALC*, *ERG*, *TLX1*, *TLX3*),[6] there is still a need for more precise molecular risk stratifications to guide adapted treatment strategies. Although some study groups utilize an initial leukocyte count above 100 000/nl as an adverse prognostic factor, the German Multicenter Study Group for Adult ALL (GMALL) applies risk stratification for T-ALL based on the immunophenotype.[7] On the basis of the results of earlier study protocols, early (sCD3^{-}, CD1a^{-}) T-ALL is regarded as a high-risk subgroup and alloSCT is recommended in first complete remission (CR).[7] Therefore, patients with ETP-ALL, being exclusively found in the subgroup of early T-ALL, are already assigned towards alloSCT within the GMALL strategy. However, the multilineage ability of normal ETPs and the stem cell-like features of oncogenetically transformed ETP-ALL may translate in primary resistance to conventional chemotherapy. Thus, a standard protocol for lymphoblastic diseases might be insufficient and new molecular targets are warranted for specific targeted therapy in order to improve overall survival (OS) in this high-risk disease.

Although the implementation of specific targeted therapies have already been applied to high-risk B-ALL characterized by *BCR-ABL* and *CRLF2/JAK* activation, alterations of the tyrosine kinase pathway have only been identified in rare cases of T-ALL presenting with rearrangements *NUP214-ABL1* and *STRN3-JAK2*.[8]

In this study, we have investigated the existence of ETP-ALL among adult patients and, in particular, assessed their clinical as well as molecular characteristics. We further explored the ETP-ALL as a molecular distinct subgroup of T-ALL and identified a high

[1]Charité, University Hospital Berlin, Campus Benjamin Franklin, Department of Hematology and Oncology, Berlin, Germany; [2]Goethe University Hospital, Department of Medicine II, Hematology and Oncology, Frankfurt/Main, Germany and [3]University Hospital Mannheim, Department of Hematology and Oncology, Mannheim, Germany. Correspondence: Dr CD Baldus, Charité, University Hospital Berlin, Campus Benjamin Franklin, Department of Hematology and Oncology, Hindenburgdamm 30, 12203 Berlin, Germany.
E-mail: claudia.baldus@charite.de
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frequency of *FLT3* mutations. Thus these findings may in future direct to innovative treatment strategies for this distinct T-ALL subgroup.

PATIENTS AND METHODS

Patients and treatment

We analyzed 178 patients classified as early T-ALL in the GMALL study trials between 1993 and 2008. The GMALL protocols include a combination of chemotherapy, radiation, and with the protocol 06/99, alloSCT was implemented for high-risk T-ALL-patients. Details of the protocols were previously described.[9] All patients gave written informed consent to participate in the study according to the Declaration of Helsinki.[10] This study was approved by the ethics board of the Johann Wolfgang Goethe-Universität Frankfurt am Main, Germany. In the GMALL study, immunophenotyping was centrally performed in the GMALL reference laboratory at the Charité University Hospital, Berlin. Immunophenotyping and subtype assignment was carried out as previously described.[11–13] High-risk T-ALL was defined by an immunophenotype of an early (sCD3$^-$, CD1$^-$) or mature (sCD3$^+$, CD1$^-$) T-ALL. ETP-ALL was defined as a subgroup within early T-ALL with the following immunophenotype: CD1a$^-$, CD8$^-$, CD5^{weak} with expression of stem cell (CD34, HLA-DR, CD117) and/or coexpression of myeloid antigens (CD13, CD33, CD65s). Absence, positivity and weak expression of antigens were defined according to the definitions in pediatric patients.[4]

Nucleic acid preparation and molecular characterization

For patients with sufficient material available, pretreatment bone marrow samples were used for DNA and total RNA extraction using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with minor modifications. Complementary DNA was synthesized using 500 ng of total RNA and avian myeloblastosis virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany) in the presence of RNase inhibitor (RNasin; Roche).

Molecular diagnostic examinations were available from in total 297 T-ALL patients (including all immunophenotypical subgroups) from the two GMALL studies 06/99 and 07/03. As far as material was available, samples were investigated by comparative real-time PCR (RT-PCR) for expression of five genes (*BAALC*, *ERG*, *IGFBP7*, *WT1* and *MN1*). mRNA expression levels for *WT1*,[14] *BAALC*[13] and *ERG*[13] were determined by RT-PCR as previously described. The primers for *IGFBP7* are available on request. *MN1*-primers were designed as reported.[15] *GUS* was used as a housekeeping gene with the exception of *ABL* in the RT-PCR for *MN1*. In 142 patient samples, the *NOTCH1* mutation status was identified by sequencing of PCR-amplified products.[16,17] For the *FBWX7* mutation status, exons 8 and 9 were sequenced in 121 patients samples as previously described.[18] *WT1* mutations analyses was performed as recently reported.[14] *FLT3* mutations (internal tandem duplications (ITD)/tyrosine kinase domain (TKD)) were analyzed using a commercially available *FLT3* mutation assay (*InVivo*Scribe Technologies, San Diego, CA, USA) in 123 T-ALL patients. Because of lack of sufficient material, not all samples could be analyzed. No significant differences regarding immunophenotype, age or sex were found in the different subsets of patients analyzed in the different experiments.

Statistical analyses

Differences in the clinical characteristics as well as response to induction therapy were tested by the Pearson χ^2-test. For OS and duration of remission in the different subgroups, Kaplan–Meier curves were created and compared by the log-rank test. OS was calculated from the time-point of study entry to the time-point of death or latest follow-up (censored). Remission duration was calculated in CR patients from the time-point of first CR until relapse or last follow-up. Stem cell transplantation in first CR, death in CR, withdrawal and continuous remission were censored at the respective dates.

The statistical difference of gene expression between two independent groups was tested by the nonparametric Mann–Whitney *U*-test. Differences in the mutation rate were analyzed by the Pearson χ^2-test. For all tests, a *P*-value <0.05 (two-sided) was considered to indicate a significant difference. All calculations were performed using the SPSS software version 17 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software version 5 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Patients' characteristics and clinical outcome

We first restricted our analysis to 178 patients diagnosed with an immunophenotype of early T-ALL who were enrolled in the GMALL trials (05/93, 06/99, 07/03) between 1993 and 2008. This cohort of early T-ALL patients represents 23% of all patients with T-ALL enrolled in the mentioned studies.[7] All samples were clearly positive for cytoplasmic CD3. Applying the immunophenotypic ETP-ALL profile (CD1a$^-$, CD8$^-$, CD5^{weak} with expression of stem cell or myeloid markers), 57 patients (32% of early T-ALL) were classified as ETP-ALL, corresponding to 7.4% of all adult T-ALL patients. Notably, none of the early T-ALL cases fulfilled the EGIL criteria for biphenotypic leukemia.

The clinical characteristics were similar between the group of ETP-ALL and non-ETP early T-ALL regarding sex, age, white blood cell count, and central nervous system involvement (Table 1). However, a lower frequency of patients with a mediastinal mass at diagnosis was found among patients with ETP-ALL compared with those with non-ETP early T-ALL (28% vs 47%; $P=0.02$). Patients of both groups were treated equally in the three GMALL trials and the rate of patients receiving an alloSCT in first CR did not differ between ETP-ALL and non-ETP early T-ALL (59% vs 60%, respectively). Slightly fewer patients with ETP-ALL achieved a CR after induction therapy (42 of 53 patients; 79%) compared with patients with non-ETP early T-ALL (93 of 113 patients; 82%;

Table 1. Characteristics of patients enrolled in the three GMALL study trials 05/93, 06/99 and 07/03 with the diagnosis of early T-ALL

	ETP-ALL (n = 57) (%)	*Non-ETP early T-ALL (n = 121) (%)*	P
Sex			
Male	47 (82.5)	84 (69.4)	0.07
Female	10 (17.5)	37 (30.6)	
Age			
15–35	27 (47.4)	68 (56.2)	0.27
36–65	30 (52.6)	53 (43.8)	
WBC count (n = 163)			
<30/nl	32 (62.7)	71 (63.4)	0.94
>30/nl	19 (37.3)	41 (36.6)	
Mediastinal mass (n = 162)			
No	37 (72.5)	59 (53.2)	0.02
Yes	14 (27.5)	52 (46.8)	
CNS involvement (n = 150)			
No	42 (91.3)	100 (96.2)	0.22
Yes	4 (8.7)	4 (3.8)	
Response to induction			
CR	42 (79.2)	93 (82.3)	0.65
PR/failure	5 (9.4)	12 (11.7)	
Death in induction	6 (11.3)	8 (7.8)	

Abbreviations: CNS, central nervous system; CR, complete remission; ETP-ALL, early T-cell precursors-acute lymphoblastic leukemia; PR, partial remission; T-ALL, acute T-lymphoblastic leukemia; WBC, white blood cell. Comparison between ETP-ALL and early T-ALL with a non-ETP immunophenotype.

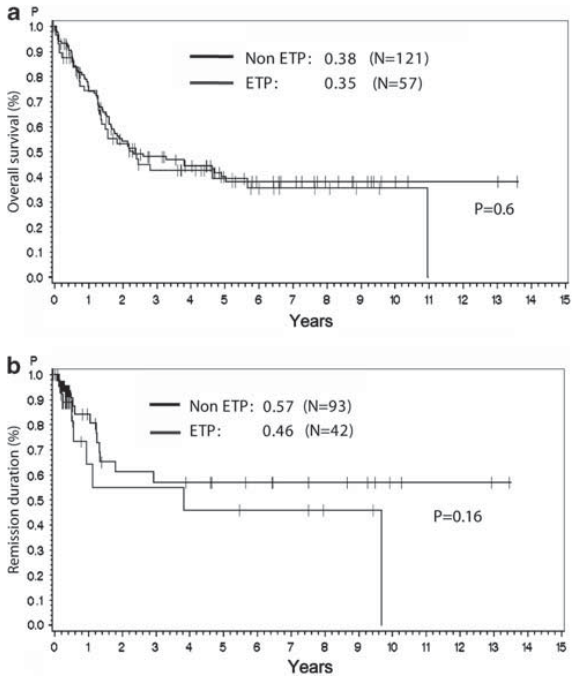

Figure 1. Kaplan–Meier analyses for OS (**a**) and remission duration (**b**) for patients with ETP-ALL and early T-ALL with a non-ETP immunophenotype. Patients were enrolled in the GMALL study trials GMALL 05/93, GMALL 06/99 and GMALL 07/03.

n.s.; Table 1). A total of 14 patients died during the induction therapy, 6 in the group of ETP-ALL, 8 in the group of non-ETP early T-ALL. Regarding all high-risk early T-ALL patients in the three study trials, the probability of survival at 10 years was comparably poor in patients with ETP-ALL and non-ETP early T-ALL (35% vs 38%, Figure 1a). In patients with early T-ALL, more patients remained in CR in the group of non-ETP early T-ALL than in the group of ETP-ALL (57% vs 46%, n.s., Figure 1b) with a follow-up of 9 years.

Aberrant gene expression in ETP-ALL

To further characterize the ETP-ALL on the molecular level, we analyzed the expression of selected genes known to be involved in the pathogenesis and with prognostic implications in T-ALL in samples with sufficient material. We first compared ETP-ALL, as a new subgroup, to all remaining T-ALL subtypes including thymic, mature and early T-ALL. As previously described, high expression of *BAALC* and *ERG* predicted an unfavorable outcome in adults with T-ALL.[10,13] Quantitative RT-PCR assays revealed that *BAALC* was 5.34-fold highly expressed in ETP-ALL compared with all remaining T-ALL ($P<0.001$, Figure 2a). *ERG* only showed a slight, however not significantly elevated expression (1.33-fold, $P=0.12$, Figure 2a). *IGFBP7* is a stem cell-associated gene, which is highly associated with *BAALC* and overexpressed in early T-ALL[19] and among the genes highly overexpressed in pediatric ETP-ALL.[4] Similar to *BAALC*, it was significantly upregulated in ETP-ALL (3.53-fold, $P=0.003$, Figure 2a). The gene *WT1* was of interest as it is widely overexpressed in AML[20] and as its overexpression is associated with a poor outcome in thymic T-ALL patients.[14] Interestingly, we found a significantly elevated *WT1* expression in ETP-ALL (4.33-fold, $P=0.04$, Figure 2a). Because of the myeloid potential of ETPs and the frequent coexpression of myeloid surface antigens in ETP-ALL lymphoblasts, we further investigated the expression of *MN1* as its overexpression was found to be associated with a shorter survival in AML without karyotypic abnormalities.[15,21] Here, we show that *MN1* was significantly overexpressed in the group of ETP-ALL compared with all remaining T-ALL patients (2.86-fold, $P<0.001$; Figure 2a).

Although these five genes were also upregulated in early T-ALL compared with mature or thymic T-ALL, *WT1*, *BAALC*, *IGFBP7* and *MN1* showed an even higher expression in the ETP-ALL group compared with the remaining group of non-ETP early T-ALL (Figure 2b).

Mutational analyses in ETP-ALL

Differences in the mutation status of candidate genes between ETP-ALL and non-ETP-ALL cases were explored (Table 2). The most frequent pathogenetic mutational event in T-ALL are *NOTCH1* mutations occurring in approximately 50–70% of the cases, predominantly in thymic T-ALL[22–24] Although *NOTCH1* mutations have been associated with an initial good response in some studies, the prognostic impact of *NOTCH1* mutations in T-ALL remains controversial.[8,25–29] In 142 adult T-ALL samples analyzed, we have found a low rate of *NOTCH1* mutations in the immature subgroup of ETP-ALL ($n=1/14$, 7.1%), whereas *NOTCH1* mutations were frequent (60.9%) in non-ETP T-ALL ($n=78/128$, $P=0.001$, Table 2). Similar findings were observed for the tumor-suppressor gene *FBXW7* involved in *NOTCH1* signaling: no *FBXW7* mutations were found in the 14 ETP-ALL samples analyzed.

Mutations in the *WT1* gene were reported in about 8% of all T-ALL patients.[14] We did not observe a significant difference in the frequency of *WT1* mutations between ETP-ALL and the remaining T-ALL (Table 2).

Although *FLT3* mutations are frequent in AML ($\sim$30%) and have important prognostic and therapeutic implications, mutations for *FLT3* as ITD or in the TKD in T-ALL are very rare.[30–32] Interestingly, in our cohort we identified seven patients with mutations of *FLT3* that were exclusive in the cohort of early T-ALL patients and predominantly found in the subgroup of ETP-ALL patients. Five cases (4.5%) had mutations in the tyrosine kinase domain of *FLT3* (D835), four of them were in the group of ETP-ALL ($P<0.001$). Another case with an *FLT3* mutation was assigned to early T-ALL, but the immunophenotype did not fulfil the criteria of an ETP-ALL due to a weak CD8 expression. This case also showed surface expression of the myeloid antigens CD13 and the stem cell markers CD34 and CD117. ITD mutations of *FLT3* were only found in two of the 123 cases, both being in the group of ETP-ALL ($P<0.001$). In total, six of seven *FLT3*-mutations found were in the group of ETP-ALL, displaying a frequency of 37.5% within this subgroup. Interestingly, *FLT3*-mutated ETP-ALL patients showed different clinical and molecular characteristics compared with *FLT3* wild-type ETP-ALL patients. The seven patients with an *FLT3* mutation had a median age of 40 years and an initial white blood cell of 3800/µl. Five *FLT3*-mutated ETP-ALL patients were transplanted in first remission and the median OS was 31.3 months with six patients being still alive. Remarkably, patients with a *FLT3* mutation showed a distinct immunophenotype with positivity for CD117 (7/7 patients), CD34 (6/7 patients), CD13 (7/7 patients), and CD2 (7/7 patients). In contrast, ETP-ALL patients with a *FLT3* wild-type status had more often positivity for CD5 (7/10 patients) and CD33 (6/10 patients).

DISCUSSION

In T-ALL, outcome has been slightly improved in the past decades, mainly because of the implementation of alloSCT for specific subgroups.[7,33] However, long-term OS only reaches rates of 30–60% depending on prognostic factors and subgroups.[34] Further improvement remains warranted and may be achieved by enhanced risk stratification and development of novel

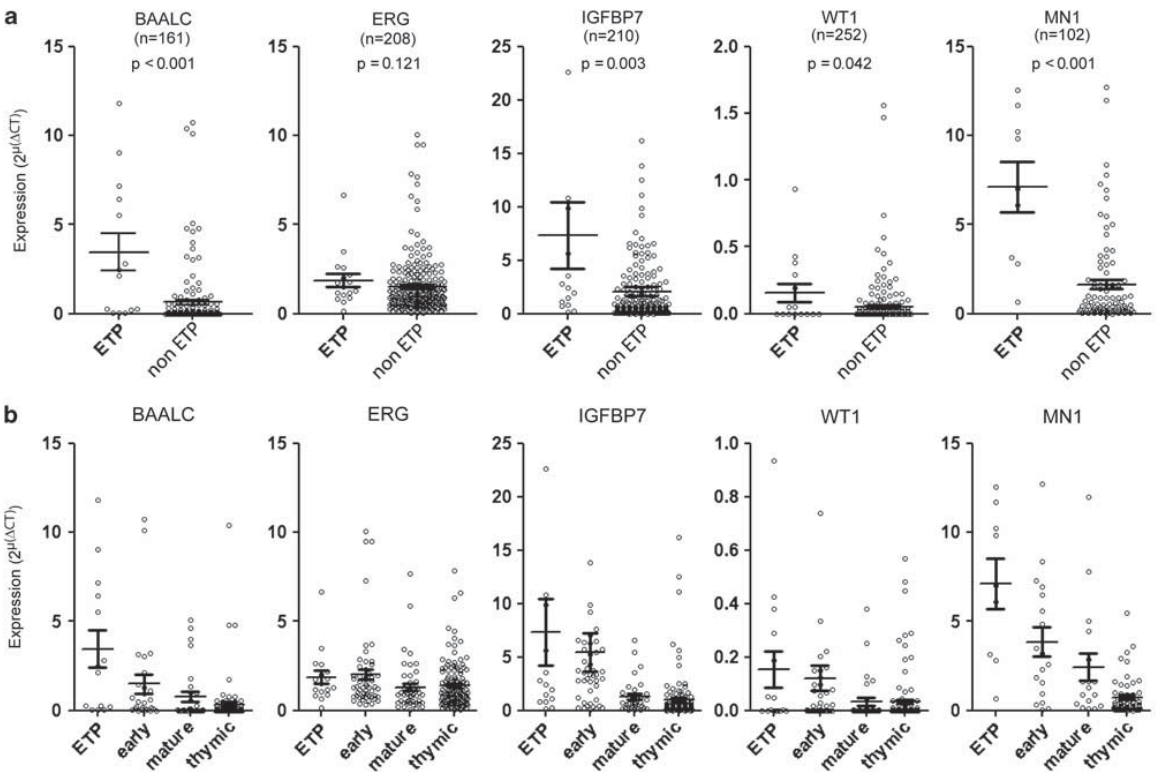

Figure 2. Expression of genes known to be correlated with outcome in T-ALL and AML. Analysis of samples with sufficient RNA was carried out by RT-PCR. For each entity the median expression is shown by a horizontal bar with the s.e. of the mean. (**a**) Comparison between ETP-ALL and the remaining non-ETP T-ALL. (**b**) Expression in the different immunophenotype-defined subgroups of T-ALL. Early T-ALL refers to non-ETP early T-ALL.

Table 2. Gene mutation status compared between (a) ETP-ALL and non-ETP T-ALL and (b) ETP-ALL and non-ETP early T-ALL

	ETP-ALL (%)	*Non-ETP T-ALL (%)*	P		*ETP-ALL (%)*	*Non-ETP early T-ALL (%)*	P
(a)				*(b)*			
FBXW7 (n = 128)				*FBXW7 (n = 50)*			
wt	14 (100)	98 (86)	0.13	wt	14 (100)	27 (100)	–
mut	0 (0)	16 (14)		mut	0 (0)	0 (0)	
NOTCH1 (n = 142)				*NOTCH1 (n = 50)*			
wt	13 (92.9)	50 (39.1)	<0.001	wt	13 (92.9)	16 (47.1)	0.003
mut	1 (7.1)	78 (60.9)		mut	1 (7.1)	18 (52.9)	
FLT3 (ITD) (n = 123)				*FLT3 (ITD) (n = 40)*			
wt	14 (87.5)	107 (100)	<0.001	wt	14 (75)	24 (100)	0.08
mut	2 (12.5)	0 (0)		mut	2 (25)	0 (0)	
FLT3 (D835) (n = 116)				*FLT3 (D835) (n = 40)*			
wt	12 (75)	99 (99)	<0.001	wt	12 (75)	23 (95.8)	0.05
mut	4 (25)	1 (1)		mut	4 (25)	1 (4.2)	
WT1 (n = 227)				*WT1 (n = 63)*			
wt	12 (80)	196 (92.5)	0.09	wt	12 (80)	42 (87.5)	0.47
mut	3 (20)	16 (7.5)		mut	3 (20)	6 (12.5)	

Abbreviations: ETP-ALL, early T-cell precursors-acute lymphoblastic leukemia; ITD, internal tandem duplications; mut, mutant; T-ALL, acute T-lymphoblastic leukemia; wt, wild type. Non-ETP T-ALL includes all thymic, mature and early T-ALL with a non-ETP immunophenotype. Analysis was performed as far as sufficient material was available.

treatment approaches based on the implementation of targeted therapies.

We characterized a new subgroup of adult patients with T-ALL (7.4%) with an immunophenotype of pediatric ETP-ALL.[4] The frequency of ETP-ALL in adult T-ALL was slightly lower than first reported for pediatric ETP-ALL, but in the range of a recent report from the Children's Oncology Group (COG).[35] Similar to the pediatric study, there were no differences in the clinical characteristics between the groups of ETP-ALL and early T-ALL with the exception of a lower frequency of a mediastinal mass in ETP-ALL.

Although many features are in common, there are relevant differences between adult and pediatric T-ALL, especially with respect to treatment. There are no immunophenotype-defined subgroups that are currently used for risk stratification in larger pediatric T-ALL trials.[36,37] In contrast, early T-ALL has been recognized as an immunophenotype-defined subtype with poor prognosis in adult T-ALL[33,] and since 1999, the GMALL study group in accordance with other study groups[38] defines early T-ALL as high-risk T-ALL[39,40] with consecutive recommendation for alloSCT. This led to an improvement in the OS at 5 years of 40%.[7] Therefore, the ETP-ALL as a subgroup of early T-ALL is already regarded as a T-ALL subgroup with poor prognosis requiring intensified therapy. In agreement with pediatric ETP-ALL, adult ETP-ALL showed an unfavorable outcome in our analysis similar to early T-ALL.[7] Interestingly, a high overlap to groups with an unfavorable prognosis, characterized by negativity of CD1 and positivity for CD33, and the group of early T-ALL is evident.[33] These T-ALLs overrepresented in early T-ALL are specifically contained in the subgroup of ETP-ALL.

To unravel the underlying molecular alterations of ETP-ALL as a specific T-ALL subgroup, we studied the expression of candidate genes reported to be of prognostic impact. Out of these, the expression of stem cell-associated genes (*BAALC* and *IGFBP7*) and genes known to be of prognostic significance in AML (*BAALC*, *MN1*, *WT1*) are underlining the immature nature of ETP-ALL. *BAALC* is preferentially expressed in the immature T-ALL phenotypes compared with the remaining T-ALL, and its high expression has been correlated to a subgroup with poor outcome.[13] Moreover, T-ALL patients with high *BAALC* expression frequently showed an aberrant expression of myeloid markers. High *BAALC* expression is also linked to an immature phenotype in AML with normal cytogenetics,[41] to a more aggressive BCR-ABL-positive acute lymphoblastic leukemia[42] and is associated with an unfavorable outcome in B-precursor ALL.[43] This led to the hypothesis that *BAALC* is a marker for acute leukemia derived from early progenitors with multi-lineage potential. Recently, *IGFBP7* was identified as a lineage-independent *BAALC* co-expressed gene with prognostic impact and a potential link to *BAALC*.[44] *BAALC* and *IGFBP7* were also found as highly expressed genes within the ETP-ALL gene expression signature.[4] In addition, overexpression of *MN1* was recently identified to be associated with ETP-ALL, and might also provide an indirect support for the poor prognosis of ETP-ALL.[45] These findings are underlined in this work as *BAALC, IGFBP7 and MN1* were upregulated in ETP-ALL, probably originating from T-cell progenitors retaining myeloid differentiation potential. This multilineage potential is further strengthened by the overexpression of the molecular marker *WT1* in ETP-ALL, a gene known to be of unfavorable prognosis in AML as well as in a subgroup of T-ALL[46-50] Interestingly, a recent work identified a subgroup in adult T-ALL (~10%) with myeloid characteristics using gene expression profiling with limited clinical data and no mutations or mutational events.[51] Up to which degree this group overlaps with ETP-ALL has to be determined in further studies.

In addition to these particular gene expression patterns, we found a clear difference in mutation events of genes known to be involved in the pathogenesis of T-ALL or AML. The high rate of *NOTCH1* mutations in T-ALL with a frequency of about 50%[26,27,52,53] makes the *NOTCH1* signaling pathway an interesting candidate for targeted therapies by the implementation of ã-secretase inhibitors.[54,55] Whether *NOTCH1* mutations could initiate leukemia alone[56-58] or are mainly secondary effects in human T-ALL[59] is discussed controversially.[60,61] Interestingly, only one *NOTCH1* mutation was found in the small group of analyzed ETP-ALL, thus demonstrating a clear pathogenetic difference to non-ETP T-ALL (61% *NOTCH1* mutations) as ETP-ALL is yet the only subgroup that lacks *NOTCH1* mutations. Additionally, in the group of ETP-ALL, mutations of the less frequent mutated tumor suppressor gene *FBXW7* were not found, again supporting a different pathogenesis for the group of ETP-ALL, likely independent from the activated *NOTCH1* pathway. Therefore, targeted therapies implementing γ-secretase inhibitors would presumably be less effective in ETP-ALL lacking mutational activated *NOTCH1* signaling.

Most remarkably, we found a high rate of *FLT3* mutations in ETP-ALL with a not yet reported high incidence of 37.5%, albeit in a small cohort (16 patients with ETP-ALL). Thus, with respect to the *FLT3*, *NOTCH1/FBXW7* and *WT1* mutation status, ETP-ALL shows a clearly different mutational profile compared with non-ETP T-ALL (Figure 3) indicating a distinct biological entity.

With respect to *FLT3* mutations, we found in contrast to AML more frequently TKD than ITD mutations. This finding is in line with data from a murine bone marrow transplantation model, where mice that received a transplant of FLT3-ITD-transduced bone marrow cells developed myeloproliferative diseases, whereas a transplant of FLT3-TKD-transduced bone marrow cells induced lymphoid disorders.[62] More recently, it was shown that a subset of common myeloid progenitors ($FLT3^+CD150^-$) has the potential to develop into T cells.[63] Although *FLT3* mutations in T-ALL are generally very rare (1–3%),[30] *FLT3* mutations were proposed to be frequently found in CD117+ T-ALL patients,[32,64] an antigen frequently expressed on ETP-ALL lymphoblasts. Although *FLT3* mutation screening is clinically not indicated in unselected newly diagnosed T-ALL, ETP-ALL as a distinct subgroup

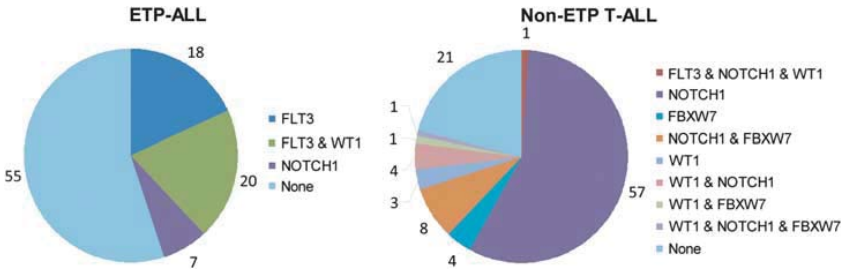

Figure 3. Distribution of mutations (*FBXW7*, *NOTCH1*, *FLT3*, *WT1*) in percent in ETP-ALL ($n = 16$ on the left) and non-ETP T-ALL ($n = 212$ on the right). Not all T-ALL samples tested for *WT1* were also tested for *NOTCH1* and *FLT3* mutations in the cohort of non-ETP T-ALL.

defined by immunocytology should now be prospectively tested for *FLT3* mutations. Moreover, these findings result in enlarged treatment options for this group with poor outcome[65] including tyrosine kinase inhibitors (TKI), which are currently investigated in trials for the treatment in AML. Importantly, similar to myeloid cells, T-ALL cell lines transfected with an *FLT3* ITD expression construct showed particular sensitivity to tyrosine kinase inhibition further justifying the use of TKI in *FLT3*-mutated ETP-ALL (data not shown). As ETP-ALL in pediatric patients is associated with high minimal residual disease levels after induction therapy,[32] the use of TKI may be efficient to lower the leukemic blast burden before alloSCT in minimal residual disease-positive patients. Furthermore, TKI could be implemented in the treatment of a relapse after alloSCT.

We identified the specific subgroup of ETP-ALL based on the definition of a certain immunophenotype in adult T-ALL. Overall, the outcome of ETP-ALL is comparably poor to early T-ALL. Therefore, intensified treatment protocols including alloSCT, as already implemented in the therapy of adult T-ALL, seems warranted for these high-risk patients. Moreover, as ETP-ALL has distinct molecular features with a high rate of *FLT3* mutations, these data point to new targeted therapy options including TKI for these high-risk T-ALL patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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6.1.2 ***Rolle der FLT3-Mutationen in der T-ALL***

(**Neumann M**, Coskun E, Fransecky L, Mochmann LH, Bartram I, Sartangi NF, Heesch S, Gökbuget N, Schwartz S, Brandts C, Schlee C, Haas R, Dührsen U, Griesshammer M, Döhner H, Ehninger G, Burmeister T, Blau O, Thiel E, Hoelzer D, Hofmann WK, Baldus CD. FLT3 mutations in early T-cell precursor ALL characterize a stem cell like leukemia and imply the clinical use of tyrosine kinase inhibitors. PLoS.One. 2013;8:e53190.)

Basierend auf den in der letzten Arbeit beschriebenen *FLT3*-Mutationen, wurden in dieser Arbeit die ETP-ALL-Patienten mit *FLT3*-Mutationen näher charakterisiert. Hierzu wurde in einer großen Kohorte von erwachsenen ETP-ALL-Patienten (n=68) der *FLT3*-Mutationsstatus erhoben. Insgesamt zeigten 24 Patienten (35%) eine *FLT3*-Mutation. Dies entspricht einer Rate an *FLT3*-Mutationen, wie sie auch in der AML beschrieben ist. Die Patienten mit *FLT3*-Mutationen zeigten einen hoch spezifischen Immunphänotyp: CD2+/CD5-/CD13+/CD33- (Spezifität: 95%, Sensitivität 88%). Während die ETP-ALL insgesamt ein deutlich alteriertes Genexpressionsprofil zur T-ALL (*BAALC*, *IGFBP7*, *WT1*, *ERG*, *MN1*, *MEF2C* hoch; *BCL11B*, *GATA3* niedrig) aufweist, bilden auch die *FLT3*-mutierten ETP-ALL-Patienten nochmals eine Subentität bezüglich ihrer Genexpression. *WT1* (0.78 vs. 0.36, p=0.003) ist höher exprimiert, während *GATA3* (0.06 vs. 5.82, p<0.001) und *IGFBP7* (0.30 vs. 2.52, p<0.001) in der Gruppe der *FLT3* mutierten Patienten im Vergleich zu den *FLT3* nicht-mutierten deutlich niedriger exprimiert werden. Des Weiteren treten *FLT3*-Mutationen exklusiv mit *NOTCH1*-Mutation auf (0/24 der *FLT3* mutierten, 10/44 der *FLT3* nicht-mutierten, p=0.01) und *FLT3* mutierte Patienten zeigen seltener ein monoklonales TCR-Rearrangement als *FLT3* nicht-mutierte Patienten (23% vs. 52%, p=0.01). Dieses fehlende TCR-Rearrangement zusammen mit der fehlenden *GATA3*-Expression legt einen frühen Differerenzierungsstop auf der Ebene des pluripotenten ETP/DN1-Stadiums zum DN2-Stadium nahe. Über die *FLT3*-Mutationen gäbe es die attraktive Therapieoption mit TKI. Um dieser weiter nachzugehen, wurden in der vorliegenden Arbeit T-ALL-Zelllinien mit FLT3-Konstrukten transfiziert. Zelllinien, die mit FLT3-ITD oder FLT3-wt-Konstrukten transfiziert wurden, zeigen sowohl einen Proliferationsvorteil gegenüber der Kontrolle als auch eine deutlich erhöhte Apoptoserate, sobald sie mit TKI behandelt werden. Insgesamt zeigt die Gruppe der *FLT3* mutierten T-ALL-Patienten bezüglich Immunphänotyp, Genexpression und Mutationsspektrum Stammzelleigenschaften und scheint einer Therapie mit TKI zugänglich zu sein.

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FLT3 Mutations in Early T-Cell Precursor ALL Characterize a Stem Cell Like Leukemia and Imply the Clinical Use of Tyrosine Kinase Inhibitors

Martin Neumann[1], Ebru Coskun[1], Lars Fransecky[1], Liliana H. Mochmann[1], Isabelle Bartram[1], Nasrin Farhadi Sartangi[1], Sandra Heesch[1], Nicola Gökbuget[2], Stefan Schwartz[1], Christian Brandts[2], Cornelia Schlee[1], Rainer Haas[3], Ulrich Dührsen[4], Martin Griesshammer[5], Hartmut Döhner[6], Gerhard Ehninger[7], Thomas Burmeister[1], Olga Blau[1], Eckhard Thiel[1], Dieter Hoelzer[2], Wolf-Karsten Hofmann[8], Claudia D. Baldus[1]*

1 Charité, University Hospital Berlin, Campus Benjamin Franklin, Department of Hematology and Oncology, Berlin, Germany, **2** Goethe University Hospital, Department of Medicine II, Hematology and Oncology, Frankfurt/Main, Germany, **3** Department of Hematology and Oncology, University of Düsseldorf, Düsseldorf, Germany, **4** Department of Hematology and Oncology, University of Essen, Essen, Germany, **5** Department of Hematology and Oncology, Klinikum Minden, Minden, Germany, **6** Department of Internal Medicine III, University of Ulm, Ulm, Germany, **7** Department of Hematology and Oncology, University of Dresden, Dresden, Germany, **8** University Hospital Mannheim, Department of Hematology and Oncology, Mannheim, Germany

Abstract

Early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) has been identified as high-risk subgroup of acute T-lymphoblastic leukemia (T-ALL) with a high rate of *FLT3*-mutations in adults. To unravel the underlying pathomechanisms and the clinical course we assessed molecular alterations and clinical characteristics in a large cohort of ETP-ALL (n = 68) in comparison to non-ETP T-ALL adult patients. Interestingly, we found a high rate of *FLT3*-mutations in ETP-ALL samples (n = 24, 35%). Furthermore, *FLT3* mutated ETP-ALL was characterized by a specific immunophenotype (CD2+/CD5-/CD13+/CD33-), a distinct gene expression pattern (aberrant expression of *IGFBP7*, *WT1*, *GATA3*) and mutational status (absence of *NOTCH1* mutations and a low frequency, 21%, of clonal TCR rearrangements). The observed low *GATA3* expression and high *WT1* expression in combination with lack of *NOTCH1* mutations and a low rate of TCR rearrangements point to a leukemic transformation at the pluripotent prothymocyte stage in *FLT3* mutated ETP-ALL. The clinical outcome in ETP-ALL patients was poor, but encouraging in those patients with allogeneic stem cell transplantation (3-year OS: 74%). To further explore the efficacy of targeted therapies, we demonstrate that T-ALL cell lines transfected with FLT3 expression constructs were particularly sensitive to tyrosine kinase inhibitors. In conclusion, *FLT3* mutated ETP-ALL defines a molecular distinct stem cell like leukemic subtype. These data warrant clinical studies with the implementation of *FLT3* inhibitors in addition to early allogeneic stem cell transplantation for this high risk subgroup.

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* E-mail: claudia.baldus@charite.de

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive leukemia accounting for 10–15% of childhood and 25% of adult ALL cases. Based on molecular studies, T-ALL can be divided into at least four molecular-cytogenetic subgroups, i.e. the *TAL/LMO*, the *TLX/HOX11*, the *TLX3/HOX11L2* and the *HOXA* subgroups [1–3]. Apart from these genetic subgroups, a fifth subgroup of T-ALL cases with developmental arrest at a very early stage of T-cell development was defined by a characteristic early T-cell precursor (ETP) signature in pediatric T-ALL [4]. This T-ALL subtype, termed as ETP-ALL, is described by an immature surface immunophenotype: absence of CD1a and CD8 expression, weak CD5 expression and expression of one or more myeloid-associated and/or stem cell-associated markers. In addition, an increased genomic instability, a high frequency of remission failures and hematologic relapse characterize this highly unfavorable T-ALL subgroup in pediatric patients [4].

Oncogenic alterations that lead to a differentiation arrest at specific stages of T-cell development are well known for specific subgroups of T-ALL. Of these, the overexpression of the orphan homeobox proteins *TLX1* and *TLX3* lead to a maturation block due to ETS1-mediated TLX recruitment to the Eα core [5]. Most recently, for the group of ETP-ALL a mutational spectrum similar to acute myeloid leukemia (AML) was observed, however no single genetic alterations could be tracked down [6]. For the majority of T-ALL, activation of *NOTCH1* signalling is a driving force in the pathogenesis [7]. Activating *NOTCH1* mutations have been found in more than 60% of T-lineage leukemias and result in a ligand-

independent release of the intracellular NOTCH1 domain, which subsequently translocates to the nucleus, where it acts as transcriptional co-activator [8–11]. Various groups have shown that activated *NOTCH1* signalling causes activation of downstream targets including *HES1, DTX1, PTCRA*, and *MYC* and clinical studies have explored gamma secretase inhibitors (GSI) as targeted therapeutic strategy in T-ALL [12–14].

In contrast to the high frequency of *NOTCH1* mutations, activating *FLT3* mutations (*FLT3*mut) occur only in a very low frequency of T-ALL cases (1–3%), but were evaluated in only limited patient series [15–17]. In contrast, mutations of the *FLT3* gene, including internal tandem duplications (ITD) and tyrosine kinase domain (TKD) mutations, are one of the most frequent somatic alterations in AML. About one third of AML patients harbor these alterations, which are associated with a poor prognosis in both, adult and pediatric AML [18,19]. These findings have promoted the use of tyrosine kinase inhibitors (TKI) in patients with *FLT3* mutated AML [20,21].

Recently, we have characterized ETP-ALL as a subgroup of early T-ALL in adults [22]. To unravel the underlying pathomechanisms of ETP-ALL and to extend our insights on *FLT3mut* ETP-ALL, we performed a comprehensive molecular and clinical study on a large cohort of adult ETP-ALL patients. We were able to demonstrate that *FLT3mut* ETP-ALL could be classified by its specific immunophenotype and distinctive stem cell like characteristics. Moreover, T-lymphoblastic cells transfected with FLT3-ITD constructs were particular sensitive to tyrosine kinase inhibition making this a new and potentially useful therapeutic option.

Materials and Methods

Patients and treatment

We screened 1241 peripheral blood and bone marrow samples of T-ALL patients that were sent to the central diagnostic reference laboratory of the German Acute Lymphoblastic Leukemia Multicenter Study Group (GMALL). Most cases were characterized with monoclonal antibodies to precursor cells (CD10, CD34, CD117, TdT and HLA-DR) and with a selection of lymphoid-associated antigens including surface and cytoplasmic (c) antigens (cCD79a, CD22, cIgM, CD19, CD20, CD24, CD3, TCR, CD2, CD5, CD4, CD8, CD7, CD1a) and myeloid-associated antigens including myeloperoxidase (MPO), CD13, CD33, CD65s, CD15, CD14, CD64. An antigen was considered positive, if they were expressed in ≥20% of leukemic cells (10% for cytoplasmic antigens). Classification of ETP-ALL was based on the immunophenotypic diagnostic criteria as originally described [4]: CD5 <75%; CD1a and CD8 <5%; CD117, CD34, HLA-DR, CD13, CD33, and CD65s >25%. CD11b was not determined (Suppl. Table S1). Of all immunophenotypically identified ETP-ALL patients (n = 142), sufficient material for further investigations was available in 68 cases. Sixteen of these 68 patients were already included in a previous work [22]. For 52 of these 68 patients clinical follow-up data were available. The median follow-up was 9.4 months (range: 0–124.6 months). Most patients were treated according to protocols of the GMALL study group (43/46, 93% by medical report, Table 1). In addition, 94 T-ALL patients from the GMALL trial 07/2003 were used as reference group, of which nine patients showed an ETP-ALL immunophenotype and were included in the cohort of 68 ETP-ALL patients [23,24]. Of the remaining 85 non-ETP T-ALL patients, 17 had an immunophenotype of early T-ALL, 15 of mature T-ALL, and 53 of thymic T-ALL. All patients gave written informed consent to participate in the study according to the Declaration of Helsinki. The studies were approved by the ethics board of the Johann Wolfgang Goethe-Universität Frankfurt/Main, Germany.

Table 1. Gene expression levels in ETP-ALL compared to non-ETP T-ALL.

Expression		ETP-ALL (N = 68)	non-ETP T-ALL (N = 85)	P-value
BAALC	median (range)	0.69 (0.0–27.1)	0.08 (0.0–160.3)	<.001
IGFBP7	median (range)	1.24 (0.01–4.2)	0.49 (0.0–16.2)	.009
WT1	median (range)	0.53 (0.0–4.2)	<0.01 (0.0–1.6)	<.001
ERG	median (range)	1.16 (0.0–18.6)	10.69 (0.5–136.7)	<.001
MN1	median (range)	4.59 (0.0–33.1)	0.66 (0.01–2.7)	<.001
BCL11B	median (range)	0.09 (0.0–1.4)	0.44 (0.0–9.9)	<.001
GATA3	median (range)	2.11 (0.0–27.3)	3.91 (0.3–32.4)	.005
MEF2C	median (range)	0.50 (0.0–5.1)	0.20 (0.0–1.7)	.001

P values were calculated by Mann-Whitney-U-test.
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Nucleic acid preparation and molecular characterization

Pretreatment bone marrow and peripheral blood samples from patients were used for DNA and total RNA extraction using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with minor modifications. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA and avian myeloblastosis virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany) in the presence of RNase inhibitor (RNasin; Roche, Mannheim, Germany).

The samples were investigated by comparative real-time PCR (RT-PCR) for expression of eight genes (*BAALC*, *ERG*, *IGFBP7*, *WT1*, *MN1*, *GATA3*, *BCL11B*, and *MEF2C*). The mRNA expression levels for *WT1* [25], *BAALC* [24], *IGFBP7* [26], and *ERG* [24] were determined by RT-PCR as previously described. *MN1*-primers were designed as reported [27]. Primer sequences for the expression analysis of *GATA3*, *BCL11B* and *MEF2C* are available on request.

The *NOTCH1* mutation status was defined by direct sequencing of the N-terminal and the C-terminal region of the HD domain, the N-terminal and the C-terminal region of the PEST domain, and the TAD domain [28,29].

WT1 mutations were analysed as recently reported [25]. *FLT3mut* (ITD/TKD) were analyzed using a commercially available FLT3 mutation assay (InVivoScribe Technologies, San Diego, USA). The TCR rearrangement status was assessed by the IdentiCloneTM TCRG Gene Clonality Assay (InVivoScribe Technologies, San Diego, USA).

Statistical analysis

Differences in the clinical characteristics were tested by the Pearson χ^2 test. For overall survival (OS) in the different subgroups, Kaplan-Meier curves were created and compared by the Log-rank test. OS was calculated from the time-point of study entry to the time-point of death or last follow-up (censored).

The statistical difference of gene expression between two independent groups was tested by the nonparametric Mann-Whitney-U-test. Differences in the mutation rates were analyzed by the Pearson χ^2 or the Fisher's exact test. For all tests a *P*-value<0.05 (two-sided) was considered to indicate a significant difference. All calculations were performed using the SPSS software version 19 (SPSS Inc., Chicago, IL, USA) and GraphPad

Prism® software version 5 (GraphPad Software Inc., LA Jolla, CA, USA).

Cell culture and chemicals

The human mature T-cell leukemia cell lines Jurkat, BE13 and MOLT-4 were obtained from the German Resource Center for Biological Material, DSMZ (Braunschweig, Germany) and previously characterized on a molecular level [30]. They were grown in RPMI media with 10% fetal bovine serum. All cell lines were cultured at 37°C in a 5% CO2 humidified chamber. TKI258 was a kind gift from Novartis (Basel, Switzerland). Tyrosine kinase inhibitors Sorafenib and PKC412 were purchased from Alexis/Enzo Life Sciences (BAY 43-9006; Loerrach, Germany) and LC Laboratories (Woburn, MA, USA) respectively. The chemotherapeutic agent cytarabine (AraC) was provided by Merck Chemicals (Darmstadt, Germany).

Plasmid constructs and transfection

For transduction of Jurkat, BE13 and MOLT-4, 2×10^6 cells were transfected with either FLT3-ITD, or FLT3-wt expression constructs and with the empty vector as a control, using the Nucleofector systems (Lonza Cologne AG, Cologne, Germany) according to the manufacturer's recommendations. The final concentration of the constructs and the empty vector control was 2 µg. FLT3-wt and FLT3-ITD expression constructs were previously described [31].

Cell proliferation assay

Cell proliferation was measured with the WST-1 reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Briefly, 48 hours (hrs) after transfection with FLT3 constructs and empty vector control, the cells were seeded in a 96-well plate with 2×10^5/well. Subsequently, the cells were cultured for 48 hrs with Sorafenib, PKC412, TKI258 and AraC as a chemotherapy agent or Dimethylsulfoxid (DMSO) as negative control. Absorbance was measured after 48 hrs by optical density absorption analyses at 450 nm using an ELISA multiplate reader.

The 50% growth inhibitory concentrations (IC_{50}) of Sorafenib, PKC412 and TKI258 were determined by plotting the logarithm of the drug concentrations (Sorafenib: 0–500 µM, PKC412: 0–18 nM, TKI258: 0–500 nM) and the growth rate of the cells treated with FLT3-ITD or FLT3-wt constructs and empty vector, using the WST-1 assay.

Apoptosis assay

The cellular apoptosis was measured transfected with FLT3-ITD or FLT3-wt constructs and with the empty vector. Briefly, after 48 hrs treatment with Sorafenib, PKC412, TKI258, and AraC cells were labelled with Annexin V and 7-amino-actinomycin D (7-AAD), using Annexin V Apoptosis Detection Kit (BD Pharmingen, Heidelberg, Germany) and then analyzed by FACS Calibur (Becton-Dickinson) to determine the percentage of apoptotic cells.

Results

Characteristics and clinical outcome of adult ETP-ALL patients

Based on the ETP-ALL specific immunophenotype, we identified pre-treatment samples of 68 newly diagnosed ETP-ALL patients. The median age was 38 years (range: 17–74 years). More patients were male (81%). Of all 68 ETP-ALL patients, follow-up data were available in 52 patients. Forty-five patients were treated according to a GMALL-like protocol, three patients to an AML-like protocol. Fifty-eight percent of patients achieved a complete remission (CR) after induction therapy (Supplementary Table S2). The cumulative 3-year OS was 60%. We further examined outcome with respect to the treatment of chemotherapy only or the allocation to allogenic stem cell transplantation (alloSCT). With the limitation of a potential selection bias for patients undergoing alloSCT, we observed for ETP-ALL patients receiving an alloSCT a favorable outcome (n = 20; 3-year OS: 74%) compared to ETP-ALL patients that were treated with chemotherapy only (n = 19; 3-year OS: 37%, P = 0.006; Figure 1). To address the potential selection bias we have also performed a landmark analysis with a time to transplant of two months. In this analyses patients undergoing alloSCT showed a favourable however not significant benefit compared to patients only receiving chemotherapy.

Aberrant gene expression and mutational analyses in ETP-ALL compared to non-ETP T-ALL

To further characterize ETP-ALL on the molecular level, we analyzed this large ETP-ALL cohort for the expression of selected genes involved in the pathogenesis and with prognostic implications in adult acute leukemia [22]. *BAALC* and *IGFBP7* were higher expressed in ETP-ALL compared to non-ETP T-ALL patients (*BAALC*, 8.6-fold, P<.001; *IGFBP7*, 2.5-fold, P = .009). Furthermore, expression levels of *WT1* and *MN1* were higher in ETP-ALL compared to non-ETP T-ALL (*WT1*, P<.001; *MN1*, 7-fold, P<.001). Additionally, expression of *MEF2C*, a gene associated with ETP-ALL [32], was significantly higher in ETP-ALL versus non-ETP T-ALL (2.6-fold, P = .001). As critical players in the differentiation program of T-lymphopoiesis, we explored the expression of the transcription factors *GATA3*, required for the development of normal ETPs [33], and *BCL11B*, necessary for the subsequent T-cell lineage commitment [34]. Both, *GATA3* and *BCL11B*, were lower expressed in ETP-ALL compared to non-ETP T-ALL (1.9-fold, P = .005; and 4.9-fold, P<.001; respectively). Similarly, ETS transcription factor *ERG* was also significantly downregulated in ETP-ALL vs. non-ETP T-ALL (9.2-fold, P<.001; Table 1) [35].

The analysis of the TCR rearrangement status revealed that 40 ETP-ALL patients (59%) lacked clonal TCR rearrangements, while 28 patients (41%) showed a monoclonal status. In contrast, 66 (78%) of non-ETP T-ALL patients showed clonal TCR rearrangements, whereas only 18 of these patients (22%) lacked monoclonal TCR rearrangements (Table 2). In addition, differences of the *NOTCH1* and *FLT3* mutation status between ETP-ALL and non-ETP T-ALL cases were explored. We found a low rate of *NOTCH1* mutations in the ETP-ALL subgroup (n = 10/68, 15%), whereas *NOTCH1* mutations were more frequent (40%) in non-ETP T-ALL patients (P<.001). In contrast, we found a high rate of *FLT3* mutations in ETP-ALL compared to non-ETP T-ALL patients: 24 of the 25 *FLT3* mutations were found in the ETP-ALL group, displaying a frequency of 35.3%, whereas non-ETP T-ALL showed a *FLT3* mutations frequency of only 1.2% (P<.001). Ten cases (6.5%) had TKD mutations, of which nine occurred in the ETP-ALL group (P = .003). ITD mutations were found in 15 cases, all belonging to the ETP-ALL group (P<.001, Table 2). In a multivariate analysis, *NOTCH1* mutation status, low expression of *BAALC*, *WT1*, *ERG*, *IGFBP7*, and TCR rearrangement had no additional prognostic impact in the subgroup of ETP-ALL.

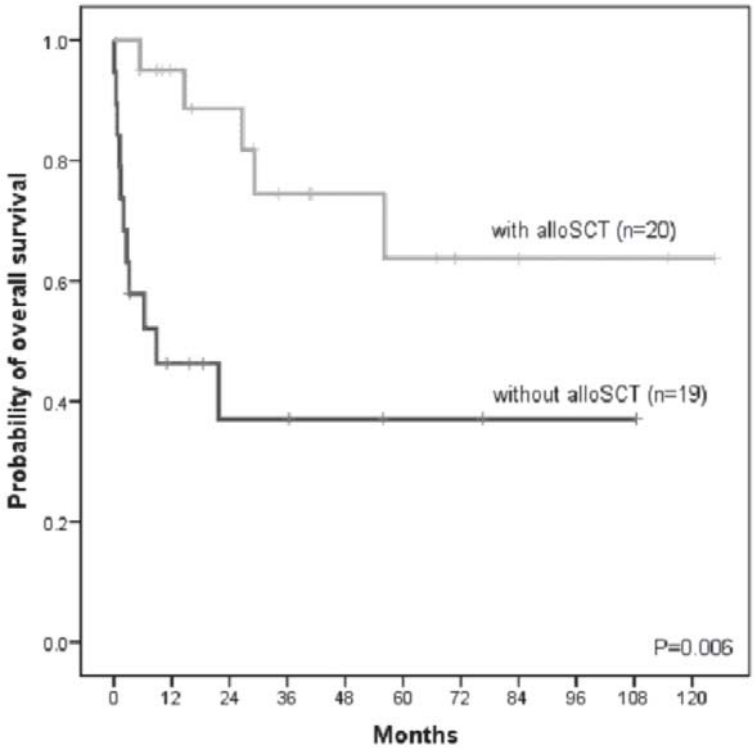

Figure 1. Kaplan Meier analysis of overall survival in adult ETP-ALL patients receiving chemotherapy only (without alloSCT) or undergoing alloSCT. P-value was calculated by the Log-Rank test. Abbreviations: alloSCT, allogeneic stem cell transplantation.
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Immunophenotype of *FLT3*mut ETP-ALL compared to *FLT3*wt ETP-ALL

In addition to the distinct immunophenotype, *FLT3*mut ETP-ALL showed specific immunophenotypic and molecular characteristics compared to *FLT3*wt ETP-ALL. In this study, 83% (20/24) of *FLT3*mut ETP-ALL patients were positive for CD117, compared to only 28% (13/44) of *FLT3*wt ETP-ALL cases (P<.001). Furthermore, *FLT3*mut ETP-ALL had a higher rate of positivity for CD2 (88% vs. 30%, P<.001) and CD13 (100% vs. 37%, P<.001) compared to *FLT3*wt ETP-ALL patients. *FLT3*wt ETP-ALL was characterized by expression of CD5 (54% vs. 4%, P<.001) and CD33 (54% vs. 4%, P<.001; Figure S1 in Supplementary Figures).

Table 2. Mutational events in ETP-ALL compared to non-ETP T-ALL.

Mutation Status		ETP-ALL	non-ETP T-ALL	P-value
TCR rearrangement	monoclonal	28 (41%)	66 (79%)	<.001
n = 153	polyclonal	40 (59%)	18 (21%)	
NOTCH1	mut	10 (15%)	35 (41%)	<.001
n = 151	wt	56 (85%)	50 (59%)	
***FLT3* total**	mut	24 (35%)	1 (1%)	<.001
n = 153	wt	44 (65%)	84 (99%)	
***FLT3* ITD**	mut	15 (22%)	0 (0%)	<.001
	wt	53 (78%)	85 (100%)	
***FLT3* D835**	mut	9 (13%)	1 (1%)	.003
	wt	59 (87%)	84 (99%)	

P values were calculated by Pearson's Chi-square test and Fisher's exact test, respectively.
doi:10.1371/journal.pone.0053190.t002

A recent study described the immunophenotype of TdT+/CD7+/CD13+/CD34+/CD117+ as highly specific for the prediction of *FLT3* mutations in an unselected cohort of T-ALL [17]. In our ETP-ALL cohort, 75% (18/24) of patients with *FLT3* mutations showed this immunophenotype, while only 7% (3/44) without *FLT3* mutations displayed this phenotype. Another *FLT3* mutation associated marker profile (sCD3-/CD117+/CD34+/CD62L+/CD56-/CD2+/CD7+/CD1a-/CD4-/CD5-/CD8-/CD13+ [36] can be adopted to 71% (17/24) of our *FLT3*mut ETP-ALL patients; this profile was highly specific for *FLT3* mutations without a false prediction. Here we established the combination of CD2+/CD5-/CD13+/CD33-, able to detect 21 of the 24 *FLT3*mut ETP-ALL patients, as highly sensitive (88%) and specific (95%) algorithm (Table 3).

Molecular characteristics of *FLT3*mut ETP-ALL in contrast to *FLT3*wt ETP-ALL

*FLT3*mut ETP-ALL showed a specific gene expression profile compared to *FLT3*wt ETP-ALL. Higher expression levels of *WT1* (2.2-fold, P = .003) and lower expression of *IGFBP7* (0.11-fold, P<.001) were characteristic for *FLT3*mut ETP-ALL. Remarkably,

Table 3. Combinations of antigens as a surrogate marker for *FLT3* mutations in ETP-ALL.

		FLT3mut (n = 24)	FLT3wt (n = 44)	P-value	Sensitivity	Specificity
CD117	pos	20	13	**<.001**	83%	70%
	neg	4	31			
TdT+/CD7+/CD13+/CD34+/CD117+[§]	pos	18	3	**<.001**	75%	93%
	neg	6	41			
CD117/CD34+/CD62L+/CD56/CD7+/CD2+/CD5-/CD13+[#]	pos	17	0	**<.001**	71%	100%
	neg	7	44			
CD2+/CD5-/CD13+/CD33-[&]	pos	21	2	**<.001**	88%	95%
	neg	3	42			

Abbreviations:
[§]combination of markers suggested by Hoehn *et al.* [17],
[#]combination of markers suggested by Paietta [36],
[&]combination of markers suggested in this paper. All combinations were adapted to the subgroup of ETP-ALL.
doi:10.1371/journal.pone.0053190.t003

*FLT3*mut ETP-ALL had a significantly lower expression of the T-cell transcription factor *GATA3* compared to *FLT3*wt ETP-ALL (P<.001). All except one *FLT3*mut ETP-ALL case had *GATA3* expression levels below the median. No significant differences were found for *BAALC*, *ERG*, *MN1*, *BCL11B*, and *MEF2C* (Table 3). As for other lymphoblastic leukemias described [37,38], *FLT3* itself is overexpressed in ETP-ALL. In this subgroup *FLT3*mut ETP-ALL showed a higher expression compared to *FLT3*wt ETP-ALL samples (P<.01, Figure S2 in Supplementary Figures).

TCR rearrangement analysis demonstrated that *FLT3*mut ETP-ALL patients predominantly lacked clonal TCR rearrangements. Only 21% of the *FLT3*mut ETP-ALL patients in contrast to 52% of the *FLT3*wt ETP-ALL patients showed a TCR rearrangement (P = .01). In addition, none of the *FLT3*mut ETP-ALL patients showed a *NOTCH1* mutation, while 23% (10/44) *FLT3*wt ETP-ALL had *NOTCH1* mutations (Table 4).

Table 4. Molecular characteristics of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients.

A Expression		FLT3mut (n = 24)	FLT3wt (n = 44)	P-value
WT1	median (range)	**0.78** (0.2–4.2)	**0.36** (0.0–3.4)	**.003**
IGFBP7	median (range)	**0.30** (0.02–4.0)	**2.52** (0.1–9.9)	**<.001**
GATA3	median (range)	**0.06** (0.0–5.7)	**5.82** (0.0–6.7)	**<.001**
BAALC	median (range)	0.44 (0.01–17.7)	0.95 (0.0–27.1)	.41
ERG	median (range)	0.94 (0.2–18.6)	1.56 (0.0–4.2)	.16
MN1	median (range)	6.35 (0.3–16.8)	7.42 (0.0–33.1)	.29
BCL11B	median (range)	0.20 (0.0–1.4)	0.05 (0.0–1.0)	.09
MEF2C	median (range)	0.71 (0.02–2.2)	0.46 (0.0–5.1)	.22
B Mutation Status				
NOTCH1	mut	0 (0%)	10 (23%)	**.01**
	wt	24 (100%)	34 (77%)	
TCR status	monoclonal	5 (21%)	23 (52%)	**.01**
	polyclonal	19 (79%)	21(48%)	

A: P-values were calculated by Mann-Whitney-U-test.
B: P-values were calculated by Pearson's Chi-square test and Fisher's exact test, respectively.
doi:10.1371/journal.pone.0053190.t004

Clinical characteristics of *FLT3* mutated ETP-ALL patients

With respect to clinical characteristics, no differences were observed between the *FLT3*mut ETP-ALL and the *FLT3*wt ETP-ALL patients regarding sex and age. The response to induction therapy was similar between both groups (CR: 13/21 vs. 13/24). Three of the 24 *FLT3*mut ETP-ALL patients were treated with an AML protocol, but none of the patients with *FLT3*wt ETP-ALL (Supplementary Table S3). The overall survival rate was similar between *FLT3*mut ETP-ALL and *FLT3*wt ETP-ALL patients (3-year survival: 58% versus 61%, P = 0.86; Figure S3 in Supplementary Figures).

Sensitivity of T-ALL cell lines transfected with *FLT3* expression constructs to TKI

In order to assess the sensitivity of TKI in a model of T-ALL with FLT3-ITD mutations, we transfected the T-ALL cell lines Jurkat, BE13 and MOLT-4 with FLT3-ITD or FLT3-wt constructs and an empty vector as control. Transfection of *FLT3* expression constructed did not alter the surface expression of myeloid (CD13, CD33) or stem cell (CD34, CD117) markers (data not shown). Cell lines transfected with FLT3-wt and FLT3-ITD constructs revealed a growth advantage compared to the empty vector transfected cells DMSO (first columns in Figure 2A–C). Treatment with TKI resulted in a selective and significant inhibition of the proliferation of FLT3-wt or FLT3-ITD transfected cells: Jurkat cells transfected with FLT3-ITD or FLT3-wt constructs showed a significant decrease in proliferation compared to empty vector transfected cells when treated with TKIs (including Sorafenib, PKC412, TKI258; Figure 2A). FLT3-ITD and FLT3-wt transfected cells were almost equally sensitive to PKC412 and TKI258, whereas empty vector transfected cells were relative insensitive. Similar results were observed for FLT3-ITD transfected MOLT-4 (Figure 2B) and BE13 cells (Figure 2C). FLT3-wt transfected cells behave different for MOLT-4 and BE13; MOLT-4 cells were more sensitive to sorafenib and BE13 cells were more resistant to PKC412 and TKI258. No differences in proliferation were observed with respect to the *FLT3* status for AraC treated cells (Figure 2A–C).

We further examined the TKI mediated apoptosis in Jurkat cells transfected with FLT3 expressing constructs. All TKIs induced enhanced apoptosis in cells transfected with FLT3 expressing constructs compared to empty vector controls (Figure S4 in Supplementary Figures). Cells treated with Sorafenib revealed a

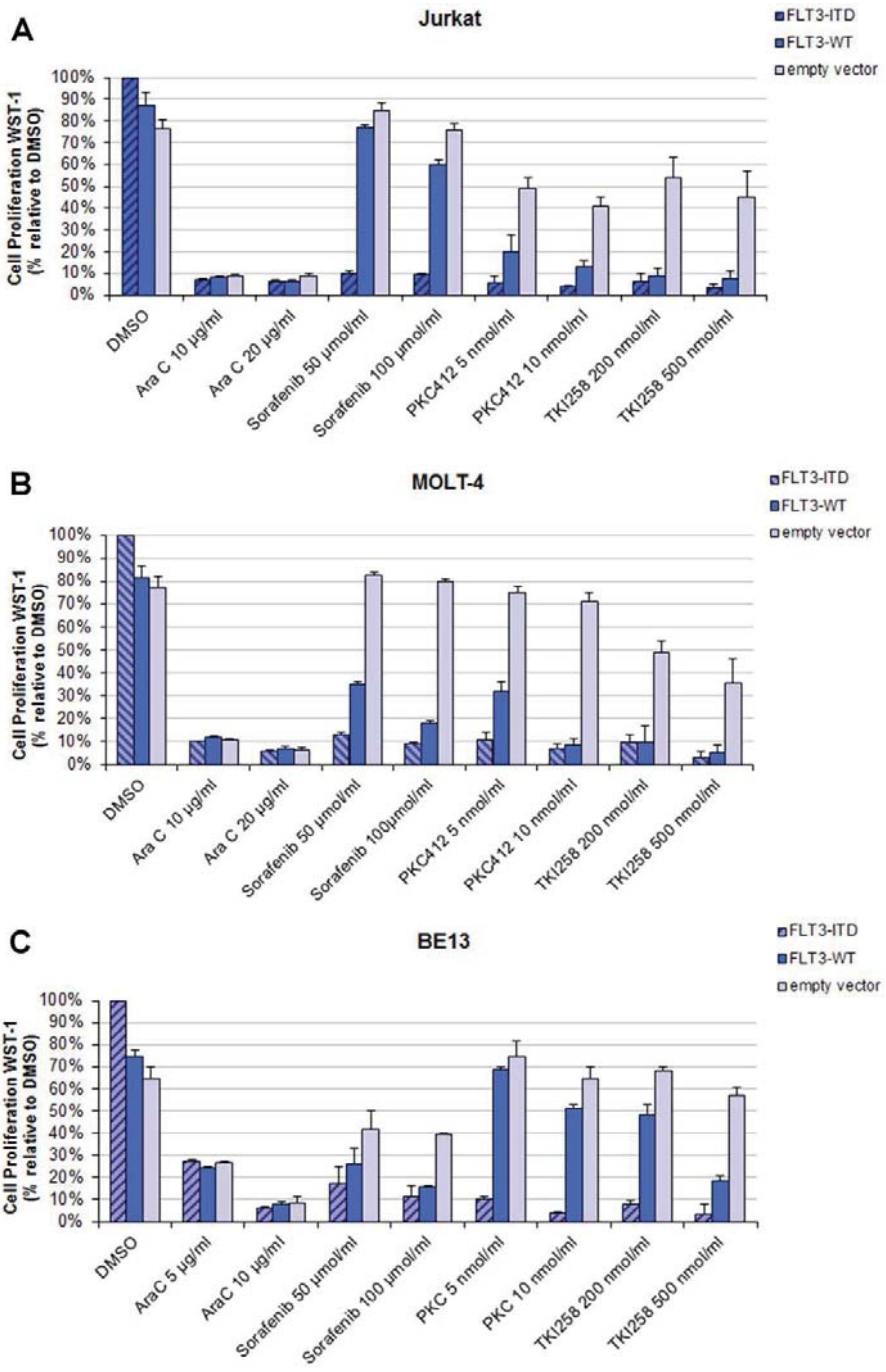

Figure 2. Effects of tyrosine kinase inhibitors on proliferation in T-ALL cell lines transfected with FLT3 expression constructs (A–C). Fourty-eight hours (hrs) after transfection, cells were seeded and cultured for additionally 48 hrs with tyrosine kinase inhibitors (PKC412, TKI258, and Sorafenib) and chemotherapy (AraC). Cell proliferation was measured using the WST-1 proliferation reagent. The mean optical density (OD) values corresponding to non-treated FLT3-ITD transfected cells were taken as 100%. The results were expressed in percentages of the OD of treated versus untreated control cells. Two experiments were performed in duplicates. For each drug two different doses were used. All results were expressed as means ±S.D. **A**: Jurkat cells. **B**: MOLT4 cells. **C**: BE13 cells.
doi:10.1371/journal.pone.0053190.g002

3-fold and 2-fold increase in apoptosis in FLT3-ITD and FLT3-wt transfected cells, respectively. Similar results were observed for PKC412 and TKI258 treated cells, whereas no significant changes in apoptosis were observed for AraC (Figure S4 in Supplementary Figures). Finally, we defined the concentration (IC50) of Sorafenib, PKC412, TKI258 and AraC that induced 50% growth inhibition of Jurkat cells (Figure 3A–D). The IC50 for Sorafenib was 25.7 μM in FLT3-ITD transfected cells, compared to to 305.5 μM and 486.5 μM in FLT3-wt, and empty vector transfected cells, respectively (Figure 3A). The IC50 for PKC412 was 2.8 nM in FLT3-ITD transfected cells, compared to 7.1 nM and 15.5 nM in FLT3-wt and empty vector transfected cells, respectively (Figure 3B). Similar growth inhibitory effects were observed for TKI258 (Figure 3C). No differences in the IC50 between the different transfected cells were seen for AraC (Figure 3D).

Discussion

In the past decades the molecular characterization of T-ALL broadly expanded and unraveled key events that drive malignant transformation. These genetic alterations may in future lead to the development and the implementation of targeted therapy. Coustan Smith et al. first identified ETP-ALL as a high risk subgroup of pediatric T-ALL characterized by a specific immature immunophenotype and a distinct gene expression profile [4]. Most recently, the genetic heterogeneity of pediatric ETP-ALL was further assessed by whole genome sequencing and next generation sequencing [6]. While various novel somatic mutations were identified, no single alteration could be detected pointing to the heterogeneous genetic background of ETP-ALL, despite the apparently common clinical and immunophenotype features. However, features shared with myeloid leukemias were present as well as mutations in genes of cytokine receptor and RAS signaling and genes involved in histone-modification were frequently observed [6]. In this work, we now focused on genes with already established prognostic and pathogenic value in AML and/or T-ALL.

We have previously characterized ETP-ALL as a high risk subgroup of early T-ALL in adults [22]. To further delineate the molecular pathomechanisms for this distinct T-ALL subgroup with stem cell like and myeloid features, we examined molecular alterations and clinical outcome in a large cohort of adult ETP-ALL patients (n = 68).

ETP-ALL is defined by a specific immunophenotype as described [4]. Recently, an additional score based on the immunophenotype was suggested to define ETP-ALL [39]. In our ETP-ALL cohort, 96% (65/68) of patients had a score greater than 6, which we used as the defining cut-off. In addition to this distinct phenotype, expression analyses of candidate genes revealed significant higher expression of stem cell associated genes and genes with adverse prognostic significance in ETP-ALL versus non-ETP T-ALL. High expression of *BAALC* and *IGFBP7*, associated with an immature high risk leukemic phenotype in adult T-ALL and AML [26,40], underscores the immature nature of ETP-ALL. Similarly, *IGFBP7*, like *MEF2C*, were also found to be significantly upregulated in pediatric ETP-ALL [4]. In addition, *MN1* identified to be associated with ETP-ALL [32], and *WT1*, a gene known to be of unfavorable prognosis in AML as well as in T-ALL in the presence of a mutation [25,41], were also overexpressed in the cohort of ETP-ALL.

We further observed distinct differences in the mutational profile: compared to non-ETP T-ALL, ETP-ALL patients showed less frequent *NOTCH1* mutations (15%). On the other hand, a

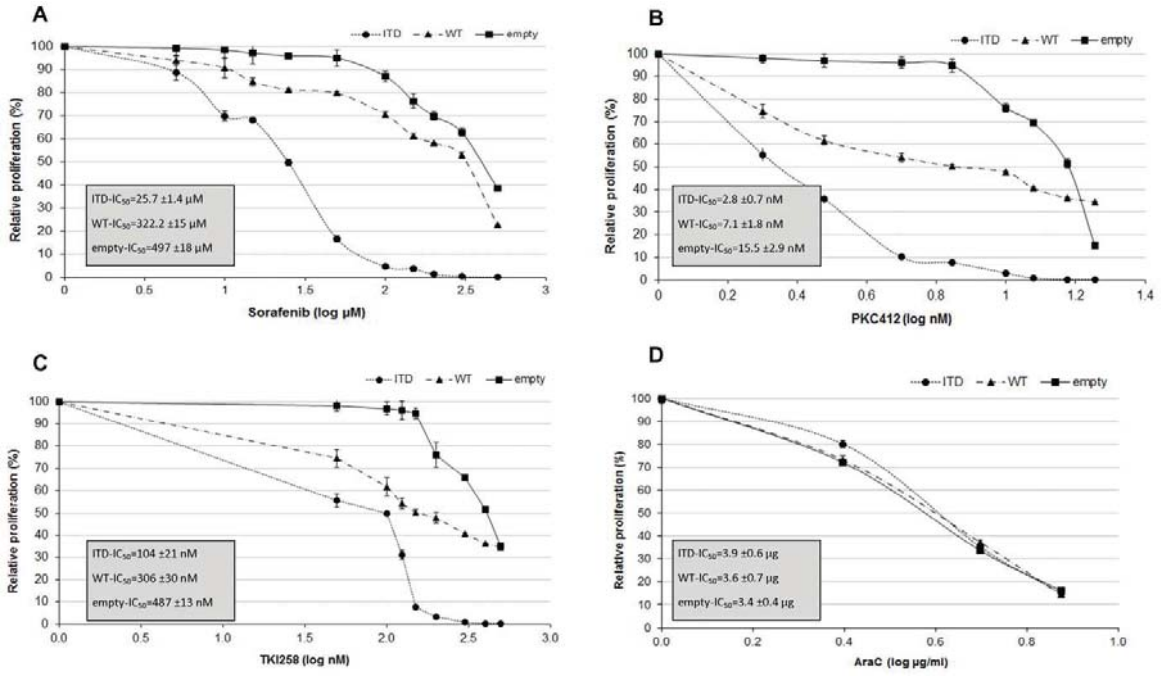

Figure 3. Growth inhibition of Jurkat cells transfected with FLT3 expression constructs (FLT3-ITD, FLT3-wt, and empty vector) and treated with Sorafenib, PKC412, TKI258 and AraC. IC_{50} was determined by WST-1 assay with different concentrations.
doi:10.1371/journal.pone.0053190.g003

high rate of *FLT3* mutations was observed in ETP-ALL (35%), contrasting the mutational profile of non-ETP T-ALL. *FLT3* mutations are one of the most frequent genetic alterations in AML [18], whereas *FLT3* is only infrequently mutated in leukemic lymphoblasts [42]. This underscores to some extent the association of ETP-ALL with early myeloid differentiation. In pediatric patients, a similar low rate of *NOTCH1* mutations (16%) was seen in ETP-ALL. *FLT3* mutations, although in a lower frequency (14%), occurred exclusively in ETP-ALL [6]. In addition, we analyzed the TCR rearrangement status in these ETP-ALL patients. In normal human T-cell development, TCR rearrangements are rare in prothymocytes, but are commonly found at the prethymocyte stage [43,44]. The frequent absence of TCR rearrangement in our cohort confirms the immaturity of ETP-ALL. Together, these data further indicate that ETP-ALL represents a distinct leukemic subtype.

Interestingly, within the ETP-ALL subgroup *FLT3*mut ETP-ALL define a new molecular stem cell entity as these cases show a specific immunophenotype and molecular characteristics compared to *FLT3*wt ETP-ALL. We observed a high expression of CD2, the myeloid antigen CD13, and CD117 in the *FLT3*mut ETP-ALL. CD117, encoded by the *c-KIT* protooncogene, is highly expressed at the early stages of hematopoietic development [45], and in acute leukemia the highest frequency of CD117 expression is found in AML [46–48]. Expression of CD117 has been associated with *FLT3* mutations in rare cases of T-ALL [15,16]. Here, in this yet largest cohort of *FLT3*mut T-ALL cases, only four *FLT3*mut patients lacked CD117 expression. Recently, combinations of surface markers were suggested as surrogate marker for *FLT3* mutations in T-ALL [17,36]. However, while these combinations yield a high sensitivity, none could detect all of the ETP-ALL cases with *FLT3*mut. In our study, a combination of CD2+/CD5-/CD13+/CD33- resulted in the highest sensitivity for the presence of *FLT3* mutations in ETP-ALL with a high specificity. For the routinely performed diagnostic flow cytometry, these combinations may help to identify ETP-ALL patients that should be tested for *FLT3* mutations.

We further observed that *FLT3*mut ETP-ALL predominantly lacked clonal TCR-rearrangements pointing to a leukemic transformation before the prothymocyte stage of T-cell development. The absence of TCR rearrangements had already been linked to early treatment failure in children with T-ALL [49], providing an indirect support for the poor prognosis of ETP-ALL. The early developmental arrest of *FLT3*mut ETP-ALL is also emphasized by the low *GATA3* expression. In normal T-cell development, GATA3 plays a definite role in the early T-lineage specification as it is required for the transformation of the ETP/DN1 to the DN2a stage [34]. Thus the leukemic transformation in *FLT3*mut ETP-ALL lacking *GATA3* expression might occur at a stem cell pluripotent prothymic stage before *GATA3* expression is induced. These data in combination with the absence of activating *NOTCH1* mutations reflect an even more immature nature of the *FLT3mut* ETP-ALL within the ETP-ALL subgroup.

ETP-ALL as a subgroup of early T-ALL reflects a high risk entity with an overall survival of approximately 50% in adults [22]. Based on the findings of the GMALL study group [50], an alloSCT should be planned in first complete remission for early T-ALL patients. Even though the selection for patients undergoing alloSCT is biased due to various confounding parameters, ETP-ALL patients receiving an alloSCT showed a remarkable favorable outcome in our cohort, whereas the outcome for ETP-ALL patients receiving chemotherapy was relatively poor. The poor response to lymphoid cell-directed ALL chemotherapy only, as already reported for pediatric ETP-ALL [4], might be due to the immature nature and myeloid characteristic of the ETP-ALL. Thus, to further improve outcome for these high risk patients, in addition to alloSCT the implementation of targeted therapies should be considered. Due to the high frequency of *FLT3* mutations in ETP-ALL, TKIs already studied in *FLT3* mutated AML [51,52] would be an attractive treatment option. We assessed the sensitivity of T-ALL cell lines transfected with FLT3-ITD and FLT3-wt expression constructs and observed that *FLT3* transfected T-ALL cells, despite of their enhanced proliferation, were particular sensitive to TKIs similar to results in AML [31]. Although the transfection of *FLT3* expression constructs in T-ALL cell lines remains an *in vitro* system, the distinct sensitivity to TKIs together with the positive experience in AML support the rational for the clinical use of TKIs in *FLT3*mut ETP-ALL. In this work, TKI side effects and the impact of TKI on the *FLT3* D835Y mutation were not evaluated. However, in analogy to AML it would be expected that the tested TKI are also able to target TKD mutations. Regarding side effects in the clinical use of TKI, the experience in AML have shown that chemotherapy backbone in combination TKIs have to be carefully chosen.

Herein, we describe that ETP-ALL patients represent a distinct molecular subgroup of adult T-ALL patients with a low frequency of *NOTCH1* mutations and a high rate of *FLT3* mutations. Moreover, we characterize *FLT3*mut ETP-ALL as a new subgroup of ETP-ALL with unique immunophenotypical and molecular features pointing to a stem cell leukemia. To further improve outcome of this high risk leukemia, targeted therapies with TKIs as well as the allocation to alloSCT should further explored.

Supporting Information

Figure S1 Expression of surface antigens comparing *FLT3*mut ETP-ALL patients and *FLT3*wt ETP-ALL patients. Median and quartiles of the percentage of positive cells in the flow cytometry are pictured. Abbreviations: * statistically significant; ns, not significant.
(DOC)

Figure S2 *FLT3* mRNA expression in 68 adult ETP-ALL samples measured by quantitative RT-PCR. The *FLT3* expression was significantly higher in *FLT3*mut ETP-ALL (n = 21) compared to *FLT3*wt ETP-ALL (n = 37) ($p<.01$).
(DOC)

Figure S3 Clinical outcome of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients. The plot shown is the Kaplan Meier analysis of overall survival. P-value was calculated by the Log-Rank test.
(DOC)

Figure S4 Effects of tyrosine kinase inhibitors on apoptosis in Jurkat cells transfected with FLT3 expression constructs. Fourty-eight hrs after transfection the cells were cultured with tyrosine kinase inhibitors (**A**: Sorafenib, **B**: PKC412, and **C**: TKI258) or **D**: AraC. Apoptosis assay was performed by Annexin V/7AAD labeling of the cells. The results are expressed in percentage of apoptotic cells. Experiments were performed in duplicates. All results were expressed as means ±S.D.
(DOC)

Table S1 Immunphenotype used for the classification of the 68 ETP-ALL patients.
(DOCX)

Table S2 Clinical characteristics of ETP-ALL patients.
(DOCX)

Table S3 Clinical characteristics of *FLT3*mut ETP-ALL versus *FLT3*wt ETP-ALL patients.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MN NG DH ET CDB. Performed the experiments: MN EC LF LM IB NFS SH SS CS OB. Analyzed the data: MN EC TB CDB. Contributed reagents/materials/analysis tools: CB RH UD MG HD GE. Wrote the paper: MN EC SS WKH CDB.

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6.2 *TEIL II: Risikostratifizierung am Beispiel der Expression von BCL11B und FAT1*

Der Identifizierung von Therapieversagern kommt insbesondere innerhalb der Standardrisikogruppe der thymischen T-ALL eine entscheidende Bedeutung zu. Potentielle Kandidaten, die über ihre Expression eine Diskriminierung erlauben, werden in den beiden folgenden Arbeiten zum Transkriptionsfaktor *BCL11B* sowie dem Protocadherin *FAT1* vorgestellt.

6.2.1 ***Bedeutung der Expression des Transkriptionsfaktors BCL11B in der T-ALL***

(Bartram I, Gökbuget N, Schlee C, Heesch S, Fransecky L, Schwartz S, Stuhlmann R, Schäfer-Eckhart K, Starck M, Reichle A, Hoelzer D, Baldus CD, **Neumann M.** Low expression of T-cell transcription factor BCL11b predicts inferior survival in adult standard risk T-cell acute lymphoblastic leukemia patients. J Hematol Oncol. 2014 Jul 15;7:51.)

Der Transkriptionsfaktor *BCL11B* ist ein entscheidender Faktor der physiologischen T-Zell-Entwicklung. Er wird während des Stadium DN2 hochreguliert und bleibt in der weiteren physiologischen T-Zell-Entwicklung transkribiert. In der vorliegenden Arbeit untersuchten wir die Expression dieses Transkriptionsfaktors, seinen Mutationsstatus sowie die prognostische Signifikanz dieser in der T-ALL des Erwachsenen. Insgesamt wurden 195 Patienten mit T-ALL untersucht, die einheitlich im Rahmen der GMALL-Studiengruppe behandelt wurden. Hierbei zeigte sich ein äußerst heterogenes Expressionsprofil von *BCL11B*. Wir konnten für die bislang größte Kohorte erwachsener T-ALL-Patienten eine hohe Mutationsrate von 14% für *BCL11B* bestimmen. Die Mutationen lagen nahezu ausschließlich in funktionell relevanten Zink-Finger-Domänen des Gens. Auch wenn *BCL11B*-Mutationen vorwiegend in der Subgruppe der thymischen T-ALL zu finden waren, zeigte sich keine Assoziation von Mutation und verändertem Überleben.

Eine andere Situation ergab die Untersuchung des Expressionsniveaus von *BCL11B* in der T-ALL. Hierbei zeigte sich ein äußerst heterogenes Expressionsprofil von *BCL11B*. Patienten mit niedriger *BCL11B*-Expression hatten nicht nur in Hinblick auf die Gesamtkohorte einen deutlichen Überlebensnachteil, sondern bemerkenswerterweise auch in der Gruppe der thymischen T-ALL, der Gruppe mit Standardrisiko. Dies sind erste Hinweise auf eine mögliche Identifizierung der Patienten mit schlechtem Therapieansprechen innerhalb dieser Gruppe.

Um Patienten mit niedriger *BCL11B*-Expression besser zu charakterisieren, analysierten wir Genexpressionsprofile (Affymetrix Hg U133 2 plus) von 86 T-ALL-Patienten. Es zeigte

sich auch in der T-ALL, dass Patienten mit hoher *BCL11B*-Expression eine Anreicherung von Genen der fortgeschritten T-Zell-Entwicklung aufwiesen. Dies spricht dafür, dass die Entdifferenzierung der T-ALL auf dem Stadium der entsprechenden physiologischen T-Zell-Entwicklung erfolgt und mag eine Erklärung für das schlechte Therapieansprechen der thymischen T-ALL-Patienten sein, die auf Transkriptionsniveau eher einer unreifen T-ALL entsprechen.

Die Studie besitzt ihre Wertigkeit neben der Charakterisierung des Transkriptionsfaktors *BCL11B* bezüglich. Mutation und Expression in der Identifizierung eines prognostischen Markers in der Gruppe der thymischen T-ALL. Dies bietet erstmals eine Möglichkeit, Patienten des Standradrisikos mit schlechtem Ansprechen auf eine konventionelle Chemotherapie zu identifizieren.

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Low expression of T-cell transcription factor *BCL11b* predicts inferior survival in adult standard risk T-cell acute lymphoblastic leukemia patients

Isabelle Bartram[1], Nicola Gökbuget[2], Cornelia Schlee[1], Sandra Heesch[1], Lars Fransecky[1], Stefan Schwartz[1], Reingard Stuhlmann[3], Kerstin Schäfer-Eckhart[4], Michael Starck[5], Albrecht Reichle[6], Dieter Hoelzer[2], Claudia D Baldus[1] and Martin Neumann[1*]

Abstract

Background: Risk stratification, detection of minimal residual disease (MRD), and implementation of novel therapeutic agents have improved outcome in acute lymphoblastic leukemia (ALL), but survival of adult patients with T-cell acute lymphoblastic leukemia (T-ALL) remains unsatisfactory. Thus, novel molecular insights and therapeutic approaches are urgently needed.

Methods: We studied the impact of *B-cell CLL/lymphoma 11b (BCL11b)*, a key regulator in normal T-cell development, in T-ALL patients enrolled into the German Multicenter Acute Lymphoblastic Leukemia Study Group trials (GMALL; n = 169). The mutational status (exon 4) of *BCL11b* was analyzed by Sanger sequencing and mRNA expression levels were determined by quantitative real-time PCR. In addition gene expression profiles generated on the Human Genome U133 Plus 2.0 Array (affymetrix) were used to investigate *BCL11b* low and high expressing T-ALL patients.

Results: We demonstrate that *BCL11b* is aberrantly expressed in T-ALL and gene expression profiles reveal an association of low *BCL11b* expression with up-regulation of immature markers. T-ALL patients characterized by low *BCL11b* expression exhibit an adverse prognosis [5-year overall survival (OS): low 35% (n = 40) vs. high 53% (n = 129), P = 0.02]. Within the standard risk group of thymic T-ALL (n = 102), low *BCL11b* expression identified patients with an unexpected poor outcome compared to those with high expression (5-year OS: 20%, n = 18 versus 62%, n = 84, P < 0.01). In addition, sequencing of exon 4 revealed a high mutation rate (14%) of *BCL11b*.

Conclusions: In summary, our data of a large adult T-ALL patient cohort show that low *BCL11b* expression was associated with poor prognosis; particularly in the standard risk group of thymic T-ALL. These findings can be utilized for improved risk prediction in a significant proportion of adult T-ALL patients, which carry a high risk of standard therapy failure despite a favorable immunophenotype.

Keywords: Adult T-cell acute lymphoblastic leukemia, BCL11b, Outcome, Standard risk, Expression, Mutation

* Correspondence: martin.neumann@charite.de
[1]Department of Hematology and Oncology, Charité, University Hospital Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany
Full list of author information is available at the end of the article

Background

Improved treatment strategies, integrating risk stratification and minimal residual disease (MRD) monitoring, have improved survival of adult patients with acute lymphoblastic leukemia (ALL) over the last decades [1,2]. Nevertheless, overall survival (OS) remains unsatisfactory with about 40-70%, depending on protocol and age group. Thus far, novel therapy approaches have mainly been developed in B-lineage ALL, where new targeted therapies with monoclonal antibodies like Rituximab or small molecule tyrosine kinase inhibitors (TKI) such as imatinib for Philadelphia chromosome/BCR-ABL-positive patients have been established [3-6]. In T-cell acute lymphoblastic leukemia (T-ALL), less progress has been made despite the introduction of nelarabine in relapsed and refractory disease [7,8]. Other molecular driven approaches, including γ-secretase inhibitors, have until now been less successful [9].

In the German Multicenter Study Group for Adult ALL (GMALL), immunologic subtypes are routinely used as prognostic factors for the risk stratification of T-ALL. Within the high risk group of early T-ALL patients the recently identified subgroup of Early T-cell precursor (ETP-) ALL has been characterized by an immature immunophenotype with a high rate of *FLT3*-mutations, suggesting a potential role for TKI in the treatment for these high risk patients [10-12]. In contrast, T-ALL patients with a thymic phenotype have a more favorable prognosis [1] and are therefore classified as standard risk. Nevertheless, this large group (56% of adult T-ALL) [3] of standard risk T-ALL contains a molecularly and clinically heterogeneous group of patients. Thus, novel insights into the molecular stratification will further aid in refining treatment options.

The *B-cell CLL/lymphoma 11b* (*BCL11b*) gene, a Krüppel-like C_2H_2 zinkfinger transcription factor located on chromosome 14q32.2, is a key player in physiologic T-cell development with potential impact on T-ALL leukemogenesis. In normal hematopoiesis, the onset of *BCL11b* expression in T-cell progenitors occurs during the onset of DN2 phase and is maintained throughout subsequent differential stages (Figure 1) [13,14]. For several target genes BCL11b acts as repressor (p21, p57), for others as activator of transcription (IL-2) [15-17]. *In vitro* studies demonstrated that knockdown inhibited proliferation and led to apoptosis in human T-ALL cell lines [18,19] and a chemo-protective effect of *BCL11b* overexpression was also observed [20].

In addition, *BCL11b* is proposed to act as haploinsufficient tumor-suppressor, as its disruption was found to be associated with lymphomagenesis in mice [21,22]. In humans, chromosomal translocations involving the *BCL11b* gene locus were identified in patients with acute myeloid leukemia (AML), T-ALL and T/myeloid acute bilineage leukemia [23-28]. Likewise, deletions and missense mutations, disrupting gene function, were reported in 9 to 16% of pediatric T-ALL patients [29,30]. One study found *BCL11b* more frequently mutated in adult patients compared to children (20% vs. 5.3%) [31], with a lower mutation rate in early immature (3.6%) and a higher rate (12%) in cortical/mature adult T-ALL [32]. Studies on the prognostic impact of *BCL11b* mutations gave diverging results: a small study reported a favorable outcome for adult T-ALL patients with *BCL11b* mutations (n = 4) [32], however, studies in pediatric patients reported no prognostic effect of mutations [29,30].

We hypothesized that deregulated expression of *BCL11b*, which is tightly regulated in normal T-cell differentiation, and *BCL11b* mutations play an important role in T-ALL. Therefore, we analyzed *BCL11b* mRNA expression levels in a large cohort of adult T-ALL patients and screened for mutations in the zinc finger region.

Results

BCL11b is heterogeneously expressed in adult T-ALL

BCL11b mRNA expression levels were not detectable in CD34 positive hematopoietic progenitor cells or unselected bone marrow (BM) samples from healthy donors, whereas high expression levels were found in CD3 positive mature T-cells (median: 2.5, Figure 2). In contrast, diagnostic BM samples of adult T-ALL patients (n = 195) showed an aberrant and highly heterogeneously *BCL11b* expression pattern (median: 0.5 range = 0-12.3; Figure 2).

As explorative approach, we divided the samples into *BCL11b*-low and *BCL11b*-high expression groups by quartiles. Expression levels in the lowest quartile Q1 (≤0.2) were regarded as *BCL11b*-low, and samples with levels in quartiles Q2-4 (>0.2) as *BCL11b*-high.

BCL11b associated global gene expression profile

To explore the underlying transcriptional profile associated with aberrant *BCL11b* expression in T-ALL, we analyzed microarray expression data of an independent cohort of 86 adult T-ALL patients [33]. Samples were classified into a low and a high *BCL11b* expression group as described in the material and methods section. We identified 229 up-regulated probe sets (corresponding to 183 unique genes, hypothetical genes/proteins and open reading frames) and 200 down-regulated probe sets (corresponding to 166 genes, hypothetical genes/proteins and open reading frames) in the *BCL11b*-low group compared to the *BCL11b*-high group (Figure 3A, Additional file 1: Table S2 and Additional file 1: Table S3). In the *BCL11b*-low group, genes reported to be suppressed by *BCL11b* were highly expressed: cyclin-dependent kinase inhibitor 1A (p21) and cyclin-dependent kinase inhibitor 1C (p57) [15,17]. Interestingly, genes associated with an immature stem cell-like phenotype were up-regulated in the *BCL11b*-low cohort including *IGFBP7*,

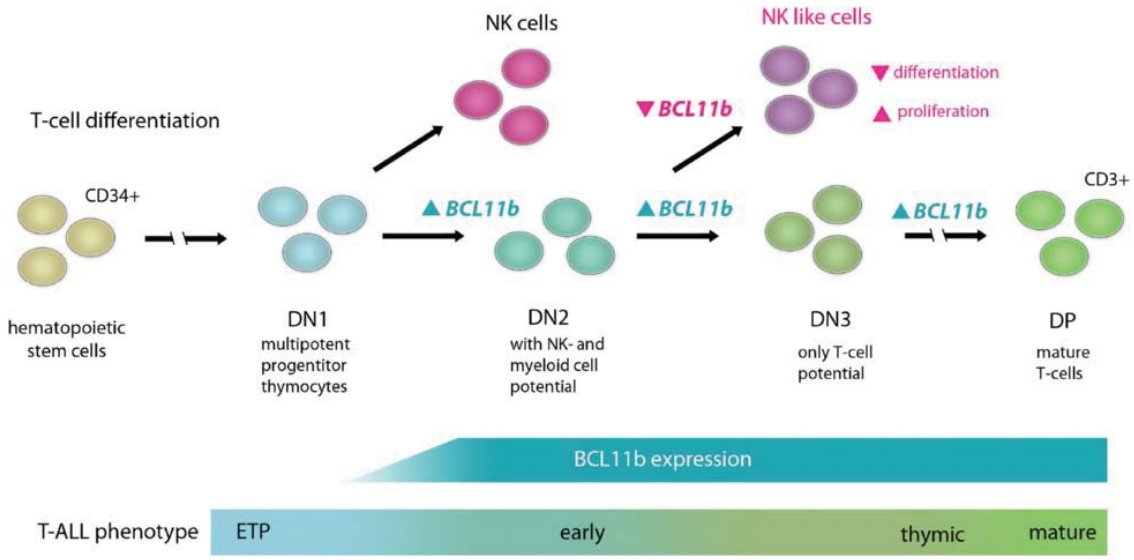

Figure 1 BCL11b expression in the stages of normal T-cell development. In the murine model BCL11b expression begins during the transition of DN1 to DN2 and continues throughout further differentiation steps and in mature T-cells [39]. Knockout at DN2 stage leads to a NK like phenotype with differentiation arrest and a high proliferative potential [13]. The corresponding human T-ALL immunophenotypes are indicated. DN = Double Negative, DP = Double Positive, NK = Natural Killer cells, ETP = Early T-cell progenitors, ▲up-regulation, ▼down-regulation.

BAALC, *CD34*, and *FLT3* [34-36]. In contrast, the *BCL11b*-high group showed co-expression of markers of a mature T-cell phenotype including several T-cell receptor genes as well as CD8 and CD6. This was further underscored in gene set enrichment analysis (GSEA), in which gene sets associated with physiological T-cell development were enriched in *BCL11b*-high ($P < 0.01$) and genes down regulated in normal T-cells were enriched in *BCL11b*-low group ($P = 0.01$; Figure 3B) [37].

BCL11b expression with respect to molecular and clinical characteristics

We further explored molecular characteristics associated with *BCL11b* expression in the T-ALL GMALL patient cohort by quantitative RT-PCR. Samples in the lowest expression quartile (Q1; n = 49; *BCL11b* expression range = 0-0.2) were compared to samples with aberrantly high *BCL11b* expression levels defined as Q2-4 (n = 146; *BCL11b* expression range >0.2-12.3, Additional file 1: Table S4). In concordance with the gene expression profiles (GEP) data, these molecular studies by RT-PCR confirmed *IGFBP7* to be overexpressed in the *BCL11b*-low compared to the *BCL11b*-high group. No significant difference between *BCL11b*-low and *BCL11b*-high patients was observed for the previously described negative prognostic factors including *BAALC*, *ERG*, and *WT1* [34,38]. *GATA3*, a transcription factor up-regulated in DN1 phase of normal T-cell development [39] was significantly lower expressed in *BCL11b*-low (median: 2.1 vs. 5.7, $P < 0.01$) compared to *BCL11b*-high patients. The frequency of TCR rearrangements was significantly lower (50% vs. 80%, $P = 0.01$) in *BCL11b*-low compared to the *BCL11b*-high group. The *BCL11b* low and the high expressing groups did not differ regarding *NOTCH1* or *WT1* mutation frequencies (Additional file 1: Table S4).

There was no difference with respect to age or sex of T-ALL patients enrolled on the GMALL 06/99 and 07/03 trials, but within the *BCL11b*-low group significantly more patients had a low white blood cell count (WBC; 62% vs. 18% $<30.000 \times 10^9$/l WBC; $P = 0.01$; Table 1). Patients with early T-ALL showed a significantly lower *BCL11b* expression (median: 0.3) compared to patients with mature (median: 0.6, $P = 0.03$) or thymic (median: 0.6, $P = 0.01$) T-ALL (Additional file 1: Figure S1).

BCL11b expression is associated with outcome in adult T-ALL

While there was no difference between *BCL11b*-low and *BCL11b*-high within the overall GMALL cohort of T-ALL patients with respect to the response to induction therapy (Table 1), the overall survival probability of *BCL11b*-low patients was significantly lower compared to *BCL11b*-high patients (Q1: n = 40, 35% at 5 yrs; Q2-4: n = 129, 53% at 5 yrs; $P = 0.02$; Figure 4A). When outcomes were analyzed separately for each quartile, T-ALL patients with *BCL11b*-low (Q1) showed an inferior outcome (Q1: n = 40; 35% OS at 5 yrs) compared to patients in the remaining quartile groups (Q2: n = 42; 52% OS at 5 yrs; Q3: n = 42;

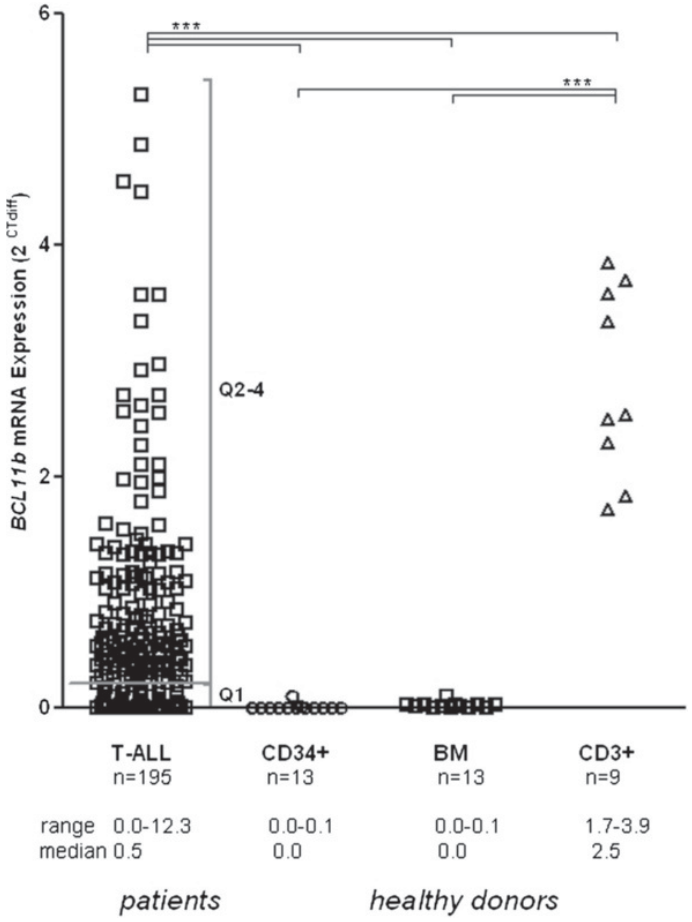

Figure 2 *BCL11b* mRNA expression in T-ALL patients and healthy donors. A heterogeneous expression pattern was observed within the T-ALL patient samples. Samples from healthy donors had a significantly higher expression of *BCL11b* in CD3 positive mature T-cells than in CD34 positive progenitor cells or unselected bone marrow (BM) samples. T-ALL patients were split into *BCL11b* expression quartiles and patients with low or lacking expression were combined in Q1 and patients with high expression in Q2-4. Outliers (n = 4 T-ALL patients, *BCL11b* expression values: 6.3, 7.6, 9.7, 12.3) were omitted from the diagram but included in the statistical analysis. BM = bone marrow; *** P < 0.01.

52% OS at 5 yrs; Q4: n = 45; 55% OS at 5 yrs; Additional file 1: Figure S2A).

Low *BCL11b* defines high risk patients within the standard risk group of thymic T-ALL

The identification of novel prognostic markers is in particular important for the largest subgroup of standard risk thymic T-ALL. As these patients are regarded as standard risk, allogeneic stem cell transplantation is not recommended in first complete remission within GMALL trials. In thymic T-ALL, *BCL11b* expression groups did not differ in the expression levels of molecular markers including *BAALC, ERG*, *IGFBP7* and *WT1*. The *BCL11b*-low group showed significantly lower expression of T-cell regulator *GATA3* compared to *BCL11b*-high (median Q1: 1.6, median Q2-4: 5.1, P < 0.01). Significantly fewer thymic *BCL11b*-low patients remained in continuous complete remission at five years compared to *BCL11b*-high thymic T-ALL patients (at 5 yrs: Q1: n = 15, 38%; Q2-4: n = 78, 72%; P = 0.02; Additional file 1: Figure S2B). Moreover, *BCL11b*-low thymic T-ALL had a significantly inferior OS compared to *BCL11b*-high thymic T-ALL patients: while only 20% (n = 18) of *BCL11b*-low patients were alive at 5 years, the 5 year OS of the *BCL11b*-high group was 62% (n = 84; P < 0.01, Figure 4B). Similar to the entire cohort, patients with thymic T-ALL *BCL11b*-low (Q1) showed a significantly inferior outcome compared to higher expression quartiles (Q1: n = 18, 20% at 5 yrs; Q2 n = 26, 67% at 5 yrs¸ Q3 n = 18, 50% at 5 yrs; Q4 n = 30, 70% at 5 yrs; P = 0.01; Additional file 1: Figure S2C).

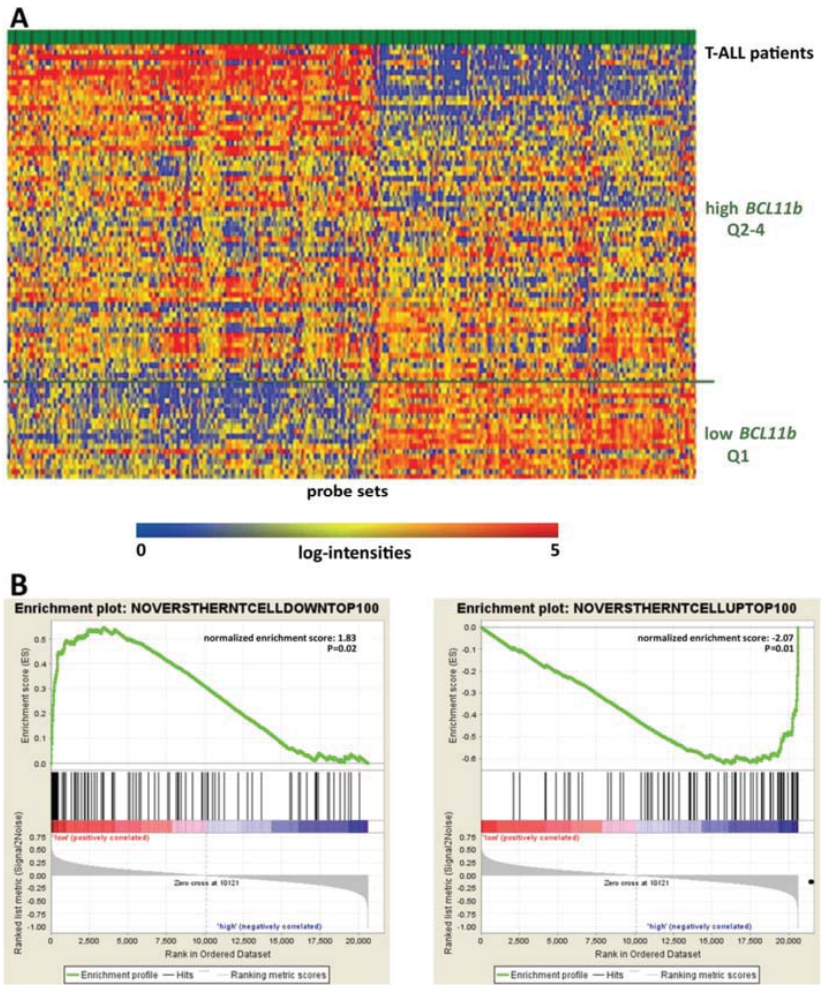

Figure 3 Gene expression profiles reveal an immature phenotype in *BCL11b-low* group. A: Heat map of two-fold or greater differentially expressed genes between *BCL11b* quartile 1 (Q1) and quartiles 2-4 (Q2-4). **B**: Gene set enrichment analysis for differentially regulated genes in physiological T-cell development according to their *BCL11b* expression. On the left side the top hundred down-regulated genes are shown, on the right side the top hundred up-regulated genes. The lists were taken from Noversthern et al. [37].

High frequency of *BCL11b* mutations in adult T-ALL

We sequenced *BCL11b* exon 4 and identified in 14% (24/178) of the T-ALL patients protein modifying alterations (Figure 5, Additional file 1: Table S6). A single T-ALL patient carried two mutations. Sixteen of the mutations were point mutations with single base pair exchanges leading to amino acid exchanges in 13 and a translation stop in three cases. Seven patients carried deletions and two insertions causing frame shifts. 23 of 24 mutations were either located within zinc finger domains or had an impact on these domains through frame shift or stop of translation, and thus have a likely impact on protein function. Nearly all patients with *BCL11b* mutations were in the *BCL11b*-high group (n = 22/23, P = 0.01). The presence of *BCL11b* mutations was associated with the maturation stage of the T-ALL. Of the 21 patients with *BCL11b* mutations, 18 were classified as thymic T-ALL (85.5%; P = 0.03; Additional file 1: Table S7). *WT1*, a negative prognostic factor, was significantly lower expressed in the *BCL11b* mutated group and other known oncogenes or molecular factors were not associated with *BCL11b* mutation status (not shown). However, outcome analyses showed no significant impact of *BCL11b* mutations and deletions on overall survival (Additional file 1: Figure S3).

Discussion

During normal T-cell development, T-cell progenitors pass through separate differentiation stages and this process is

Table 1 Clinical characteristics of GMALL T-ALL patients with respect to *BCL11b* expression

Characteristics	*BCL11b*-low	*BCL11b*-high	
	Q1	Q2-4	P-value
n	40	129	
BCL11b expression			
Median	0.1	0.8	
Range	0-0.2	0.2-12.3	
Age			n.s.
15-35 yrs	27	70	
36-55 yrs	13	48	
56-65 yrs	0	11	
Sex			n.s.
Female %	20%	26%	
WBC, $\times 10^9$/l			0.01
<30,000	24	42	
30-100,000	10	53	
>100,000	5	30	
T-ALL subtype			0.01
Early n (%)	17 (42%)	23 (18%)	
Mature n (%)	5 (13%)	22 (17%)	
Thymic n (%)	18 (45%)	84 (65%)	
Response to induction therapy			n.s.
CR n (%)	37 (95%)	115 (94%)	
ED n (%)	1 (3%)	3 (2%)	
Failure n (%)	1 (3%)	5 (4%)	

Abbreviations: *CR* = complete remission, *ED* = early death, *WBC* = white blood cell count.

tightly regulated by lineage specific transcription factors. While onset of *GATA3* and *TCF-1* expression characterize the early DN1 (ETP) phase in T-cell differentiation, *BCL11b* is expressed at the later DN2 stage [39]. As a gatekeeper of T-cell development, it is essential for the correct αβ T-cell development [3,40]. Loss of *BCL11b* at various stages was shown to induce a natural killer cell-like phenotype with a differentiation arrest and was associated with a high proliferative potential [14,40]. In addition, *BCL11b* is necessary for T-cell identity maintenance. Alteration of different stage-specific transcription factors and key regulators of T-cell differentiation by silencing, ectopic expression or mutations have shown to contribute in oncogenic transformation in T-ALL. For example, aberrant *NOTCH1* signaling through activating mutations found in 50-60% of T-ALL cases [41,42], is a prominent example of a potent driver event in T-cell leukemogenesis. For *TAL1*, which plays a key role in hematopoietic stem cell development, rearrangements and aberrant expression were found in T-ALL patients [43]. In this case, *TAL1* expression was positively correlated with survival in pediatric patients [44]. Likewise for the homeobox transcription factor *TXL-1* deregulation and gene locus abnormalities had been associated with improved outcome for *TXL*-1-high T-ALL patients [45]. Understanding of the molecular processes involved in T-ALL pathology offers the possibility to refine prognosis and stratify therapeutic algorithms.

For *BCL11b* aberrant expression levels, deletions and mutations have been reported in T-ALL [29-32,46,47]. Here we comprehensively investigated the implications of altered *BCL11b* expression and loss of function mutations in a large cohort of adult T-ALL patients (n = 169). While $CD34^+$ hematopoietic progenitor cells and unselected BM samples of healthy donors lack *BCL11b* expression, T-ALL patients showed an aberrant and highly heterogonous *BCL11b* expression pattern. Similar to normal T-cell differentiation, the expression of *BCL11b* reflected the maturation stage in T-ALL and thus was significantly lower in the early compared to thymic and mature T-ALL subgroups (Figure 1, Additional file 1: Figure S1). Analysis of microarray expression data confirmed this observation revealing up-regulation of genes associated with an immature phenotype in *BCL11b*-low and an enrichment of markers of a mature T-cell phenotype in *BCL11b*-high T-ALL.

While the combined patient data suggested that *BCL11b* expression reflects the differentiation arrest of leukemic cells, expression was heterogeneously distributed and patients that lacked or had very low *BCL11b* expression were found in all immunophenotypic subgroups. This suggests that *BCL11b* is not just a mere marker of genetically more differentiated blasts, but may act as a maturation dependent tumor suppressor, which is supported in other studies [29,30,47]. If deregulated during differentiation, disruption of normal *BCL11b* function may contribute to malignant transformation.

In this study the cohort of uniformly treated adult T-ALL patients, the *BCL11b*-low subgroup had a significantly inferior OS compared to the *BCL11b*-high patients. In particular, in the standard risk group of thymic T-ALL, *BCL11b* was of relevant prognostic impact: 62% of *BCL11b*-high patients were alive after 5 years, whereas survival was only 20% at 5 years in the *BCL11b*-low subgroup. Remission duration was also significantly shorter for patients within the *BCL11b*-low subgroup. This contrasts a study in pediatric T-ALL patients, which showed that *BCL11b* expression had no impact on OS [30], although the difference may be due to the smaller sample size of the study and that patients were not classified into immunophenotypic subgroups. Nevertheless, low *BCL11b* expression was associated with chemotherapy induction failure in the same study.

While immunophenotypic classification of T-ALL has improved outcome prediction, a relevant percentage of patients classified as standard risk based on their thymic

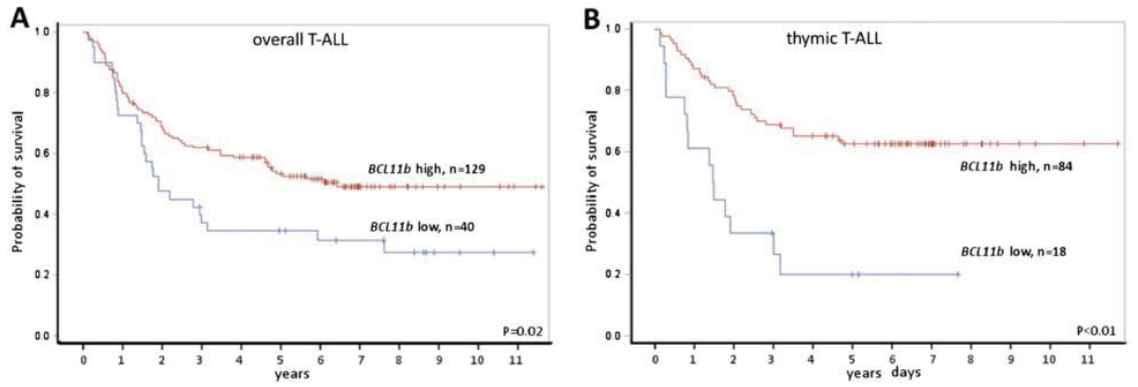

Figure 4 Kaplan–Meier analyses of overall survival (OS) and remission duration in T-ALL with respect to *BCL11b* mRNA expression. **A**: T-ALL patients with low *BCL11b* expression showed significant inferior OS than patients with higher expression (low expression quartile Q1 vs. high Q2-4 expression quartiles; P = 0.02; log-rank test). **B**: The *BCL11b* low expressing thymic T-ALL patients had a highly significant inferior OS (P < 0.01).

immunophenotype, fail conventional chemotherapy. As thymic T-ALL on the molecular and clinical levels compromises a highly heterogeneous cohort, it remains essential to identify patients with a high risk of relapse to adjust treatment stratification. Our results indicate that thymic *BCL11b*-low T-ALL patients represent a high risk subgroup which would benefit from intensified MRD monitoring and treatment stratification including allogeneic stem cell transplantation. Since lack of *BCL11b* expression proved to indicate inferior survival, we investigated disruption of the gene's function on the mutational level. *BCL11b* mutations in pediatric and adult patients had been reported in T-ALL in the zinc finger structures of exon 4 [29,30,32,46]. In agreement with these studies, we discovered a high rate (14%) of *BCL11b* mutations in this large cohort of T-ALL patients. We found an association of immunophenotype and mutation frequency: only 2% mutational rate in early T-ALL patients and 19% mutational rate in thymic T-ALL patients. Our results support a recent study in which adult T-ALL patients characterized as "early/immature" had fewer *BCL11b* mutations [32]. The number of *BCL11b*

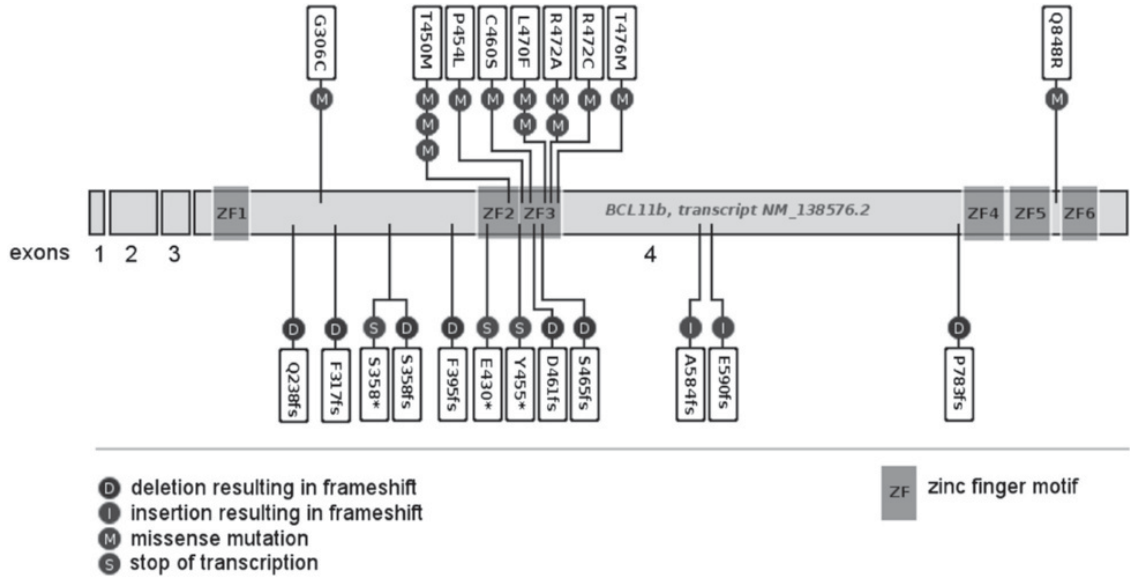

Figure 5 Mutations found in exon 4 of *BCL11b* in T-ALL. Mutation analysis was performed by Sanger Sequencing in 178 T-ALL patients, 14% of patients were revealed to harbor protein changing mutations in exon 4 of *BCL11b*.

mutations in thymic T-ALL samples in this report was low, limiting the statistical significance regarding outcome. Also, gene expression studies may be more sensitive to identify patients with the specific outcome-associated phenotype caused by *BCL11b* down-regulation.

Further studies are needed to fully understand the biological relevance of *BCL11b* mutations, and to explore potential directed therapies.

In summary, we identified *BCL11b* expression as a potent prognostic factor in the overall cohort and in particular in the standard risk subgroup of thymic T-ALL. These findings will help to identify patients with an enhanced risk of failure to standard therapy, however, standardized detection analyses of aberrant gene expression levels in a routine diagnostic setting remains challenging. More importantly, alterations in critical transcription factors contribute to leukemogenesis and may be regarded as ideal candidates for differentiation directed therapies in the future.

Methods

Patients

We analyzed diagnostic BM material of 195 adult T-ALL patients sent to the GMALL reference laboratory [48]. Immunophenotyping of the samples was centrally performed in the GMALL reference laboratory at the Charité, University Hospital Berlin, Germany, as previously described [49,50], and classified into the T-ALL subgroups: early (n = 50), mature (n = 33) and thymic (n = 112). Of these, 169 T-ALL patients were consecutively enrolled in the GMALL trials 06/99 and 07/2003 [51] with available clinical follow-up data. Additionally, samples of healthy adult donors were used, 13 BM samples, 13 CD34+ progenitors and nine CD3+ selected T-cell samples. Written informed consent according to the declaration of Helsinki had been given. Studies were approved by the ethics board and registered in a public registry (clincaltrials.gov NCT00199056, NCT0098991).

Sample preparation and qRT-PCR

Total RNA and DNA extractions were performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To analyze *BCL11b* expression, complementary DNA was synthesized and quantitative real-time polymerase chain reaction (qRT-PCR) was performed, using *Glucose-6-Phosphate Isomerase* (*GPI*) as internal control, as previously described [52]. For *BCL11b* amplification forward primer AACCCGCAGCACTTGTCC, reverse-primer ATTT GACACTGGCCACAGGT and probe FAM-CTCATCAC CCCAGAGGCTGACCAT-BHQ1 spanning exons 1 and 2 were used. Expression levels of *BCL11b* were calculated using the mean of ΔCT from two replicates and expressed as $2^{\mu(\Delta CT)}$. The mRNA expression levels for *IGFBP7*, *WT1*, *BAALC*, *ERG*, and *GATA3* by qRT-PCR, as well as mutation status of *WT1* and *NOTCH1* and TCR-rearrangements had been determined in previous studies [12,34,35,52-54].

Gene expression profiles

BCL11b-associated GEP of an independent set of 86 adult T-ALL samples were generated from raw data obtained from the Microarrays Innovations in Leukemia (MILE) multicenter study (HG-U133 Plus 2.0; Affymetrix, Santa Clara, CA, USA) [33]. For the GEP-analysis, samples were divided into quartiles (Q) according to *BCL11b* expression [median of the two probe sets (219528_s_at, 222895_s_at)]. To identify *BCL11b*-associated GEP signatures, the lowest expression quartile (Q1) was compared to quartiles 2 to 4 united (Q2-4). Lists of genes with a 2-fold under- or over-expression were generated. Statistical significance was calculated by the non-parametric *t*-test with a *P*-value ≤0.05. The data analyses were carried out with GeneSpring software version 4.2 (Silicon Genetics, Redwood City, CA, USA).

Mutational analysis

Quantity and quality of genomic DNA was sufficient for the mutational analysis of *BCL11b* in 178 T-ALL samples. As previous studies had detected only very few mutations outside exon 4, which harbors all six of the gene's zinc finger domains, we focused on this region [29,30]. Primer pairs were newly designed or used as previously published for bidirectional Sanger sequencing of exon 4 (complete list see Additional file 1: Table S1) [29]. Geneious version 5.4.3 software (Biomatters Ltd., Auckland, NZ) was used for analysis.

Additional file

Additional file 1: Statistical analysis. Figure S1. BCL11b mRNA expression in T-ALL immunophenotypic subtypes. **Figure S2.** Kaplan–Meier analysis of overall survival (OS) in T-ALL with respect to BCL11b mRNA expression. **Figure S3.** Kaplan–Meier analysis of overall survival (OS) in T-ALL with respect to BCL11b mutation status. **Table S1.** Primer sets designed for human BCL11b exon 4 (NP_612808.1). **Table S2.** Probe sets up-regulated in the BCL11b-low group of T-ALL patients. **Table S3.** Probe sets up-regulated in the BCL11b-high group of T-ALL patients. **Table S4.** Molecular characteristics of T-ALL patients with respect to BCL11b expression. **Table S5.** Molecular characteristics of thymic GMALL T-ALL patients in respect to BCL11b expression. **Table S6.** BCL11b exon 4 mutations in T-ALL. **Table S7.** Clinical characteristics of GMALL T-ALL patients in respect to BCL11b mutations. **Table S8.** Members of the German Multicenter Study Group for Adult ALL Supplemental Methods: Statistics. References.

Abbreviations

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; BM: Bone marrow; DN: Double negative; ETP-ALL: Early t-cell precursor ALL; GEP: Gene expression profile; GSEA: Gene set enrichment analysis; GMALL: German multicenter acute lymphoblastic leukemia study group; MILE: Microarrays innovations in leukemia; MRD: Minimal residual disease; OS: Overall survival; qRT-PCR: Quantitative real-time polymerase chain reaction; T-ALL: T-cell acute lymphoblastic leukemia; TKI: Tyrosine kinase inhibitors.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
IB performed laboratory work, data analysis and wrote the manuscript. NG supervised the GMALL study center, performed statistical analysis and reviewed the manuscript. CS performed laboratory work for this study. SH helped to design the study. LF provided expression data. SS, RS, KSE, MS, AR and DH recruited the study patients and performed the study procedures. CDB coordinate the research and reviewed the manuscript. MN performed statistical analysis and reviewed the manuscript. All authors read and approved the final manuscript.

Author details
[1]Department of Hematology and Oncology, Charité, University Hospital Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany. [2]Department of Medicine II, Hematology/Oncology, Goethe University Hospital, Frankfurt/M, Germany. [3]Department of Hematology, Oncology and Stem cell transplantation, Asklepios Klinik St. Georg, Asklepios Klinik St. Georg, Hamburg, Germany. [4]Department of Hematology and Oncology, Klinikum Nürnberg, Nürnberg, Germany. [5]Department of Hematology and Oncology, Klinikum Schwabing, München, Germany. [6]Department for Internal Medicine III, University Hospital Regensburg, Regensburg, Germany.

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6.2.2 ***Charakterisierung von Mutationen und Expression des Cadherins FAT1 in der T-ALL***

(**Neumann M**, Seehawer M, Schlee C, Vosberg S, Heesch S, von der Heide EK, Graf A, Krebs S, Blum H, Gökbuget N, Schwartz S, Hoelzer D, Greif PA, Baldus CD. FAT1 expression and mutations in adult acute lymphoblastic leukemia. Blood Cancer J. 2014 Jun 27;4:e224.)

FAT1 codiert ein großes Cadherin-Protein, welches insgesamt 34 Cadherin-Domänen besitzt (Katoh, 2012). Es ist auf Chromosom 4q34-35 lokalisiert, einer Region, die regelmäßig in mehreren Krebsarten deletiert ist (Morris *et al*, 2013). Die funktionelle Charakterisierung zeigte eine Assoziation zum WNT-Signalweg (Morris *et al*, 2013). Obwohl *FAT1* erstmals in einer T-ALL-Zelllinie beschrieben wurde, gab es bis vor kurzem keine weitergehenden Untersuchungen dieses Gens in akuten Leukämien. Erst durch die Detektion von rekurrenten somatischen Mutationen in der ETP-ALL (Neumann *et al, 2013*) und als potentieller MRD-Marker in der ALL (Campana *et al*, 2010)) rückte FAT1 wieder in den Fokus weiterer Untersuchungen.

Basierend auf einer Arbeit, die in einer „in silico"-Arbeit der Expression von FAT1 prognostischen Wert in der pädiatrischen ALL zuwies (De Bock *et al*, 2012), untersuchten wir eine große Kohorte im Rahmen der GMALL-Studiengruppe einheitlich behandelter ALL-Patienten, sowohl Patienten mit BCP-ALL als auch mit einer T-ALL, auf Expression und prognostische Signifikanz von *FAT1*. Wir fanden eine hohe Anzahl von Patienten mit aberrant erhöhter Expression von *FAT1*: 61 von 112 Patienten (54%) mit einer T-ALL und 41 von 129 Patienten (32%) mit einer BCP-ALL zeigten erhöhte Expressionswerte für *FAT1*. Hierbei waren in der T-ALL eher reife Subgruppen stärker betroffen (thymische T-ALL: 74%; reife T-ALL: 45%, frühe T-ALL: 4%) Dies steht in scharfem Kontrast zur Untersuchung verschiedener Zellpopulationen (Knochenmark, CD34+-Stammzellen, peripheres Blut, CD3+-T-Zellen) von gesunden Probanden, die allesamt ein Expressionsniveau unter der Nachweisgrenze aufwiesen. Einzig in mesenchymalen Stammzellen konnte eine Expression von *FAT1* nachgewiesen werden. Diese isolierte Expression in onkogen transformierten Zellen macht *FAT1* nicht nur einen Kandidaten für ein MRD-Monitoring, sondern macht es auch als therapeutischen Angriffspunkt interessant.

Die in der Literatur beschriebene prognostische Wertigkeit konnten wir in unserer größeren Kohorte nicht nachvollziehen. Hierfür mögen neben den Problemen einer „in silico"-Analyse die Unterschiede zwischen pädiatrischen und erwachsenen ALL-Patienten als auch die Kohortengröße und-zusammenstellung eine Rolle spielen.

Zusätzlich fanden wir in einem signifikanten Anteil der T-ALL-Patienten (8/68; 12%) Mutationen des *FAT1*-Gens. Die Gruppe der *FAT1*-mutierten Patienten unterschied sich weder in klinischen Parametern noch im Expressionsniveau noch in Überlebensdaten von Patienten ohne eine *FAT1*-Mutation.

Zusammengenommen bleiben hierdurch Zweifel an der direkten Bedeutung von *FAT1* in der Leukämogenese. Allerdings ist auf Grund des exklusiven aberranten Expressionsniveau, der gehäuften somatischen Mutationen auch in anderen Krebsentitäten (z-B- Kopf-Hals-Tumore), zusätzlichen Mutationen der homologen Gene *FAT2*, *FAT3* und *FAT4* und der Verknüpfung mit dem WNT-Signalweg (Stransky *et al*, 2011) von einer zentralen Rolle des Cadherins in der Krankheitsentstehung oder als Antwort auf die Krankheit auszugehen. Dies ist zurzeit Gegenstand weiterer funktioneller Untersuchungen.

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LETTER TO THE EDITOR

FAT1 expression and mutations in adult acute lymphoblastic leukemia

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The cadherin gene *FAT1*, located on chromosome 4q34-35 (ref. 1) within a region frequently deleted in human cancers,[2] encodes a large protein with 34 extracellular cadherin repeats.[3] In solid tumors, aberrant expression of *FAT1* was found to be associated with disease progression.[4] Although the gene was originally cloned from a human T-cell acute lymphoblastic leukemia (T-ALL) cell line,[4] *FAT1* just recently gained interest owing to its altered gene expression levels and the detection of somatic mutations identified by next-generation sequencing (NGS) in acute leukemia.[2,5–7] *FAT1* was shown to be aberrantly expressed in pediatric patients with acute leukemia, whereas hematopoietic progenitors from healthy donors lacked *FAT1* expression.[5,8] In addition, a recent report correlated high *FAT1* expression with a higher probability of relapse in a small cohort of pediatric patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) based on an *in silico* analysis comprising two BCP-ALL data sets including 32 and 27 patients.[5]

With the emergence of NGS, it has become obvious that *FAT1* is not only aberrantly expressed in various tumors, but also frequently mutated in solid tumors.[7,9] Morris *et al.*[2] were able to link the mutational inactivation of *FAT1* to the loss of its tumor suppressor capacity and the activation of the WNT pathway. In summary, these data make *FAT1* an interesting candidate for disease monitoring, risk stratification and the development of targeted therapies. Herein, we investigated *FAT1* expression in a large, homogenously treated cohort of adult acute leukemia patients, and explored the mutation status of *FAT1* and its clinical significance.

We analyzed *FAT1* expression by real-time PCR in different cell populations of healthy donors, various leukemia cell lines, a small cohort of acute myeloid leukemia (AML; $n=13$), in 112 adult T-ALL samples and in 129 adult BCP-ALL samples (Supplementary Methods). We examined the clinical and molecular characteristics with respect to *FAT1* expression in this large cohort of adult ALL patients using specimens sent to the reference laboratory of the German Study Group for adult ALL (GMALL; $n=231$). Of these, 180 patients were enrolled into the trials GMALL 06/99 and 07/03 with available clinical follow-up. The treatment strategy of the GMALL trials has been described previously (Supplementary Methods). We were able to confirm the reported expression pattern for *FAT1* in different cell lines (Supplementary Figure S1).[5] The cell line BE13 showed the lowest, nearly absent, expression of *FAT1* and was used as a cutoff to define samples with a high expression (*FAT1*pos) compared with a lower/absent expression (*FAT1*neg). We also investigated the expression of *FAT1* in different cell populations from healthy donors. Unselected bone marrow (BM), $CD34^+$ progenitors, peripheral blood and $CD3^+$ T cells from healthy donors lacked *FAT1* expression (Supplementary Figure S2), whereas *FAT1* expression was highly expressed in BM-derived mesenchymal stromal cells (BMSC) from healthy donors (Supplementary Figure S2). In contrast, *FAT1* was aberrantly expressed in adult leukemia: 23% of AML and 32% of BCP-ALL patients expressed *FAT1* and were defined as *FAT1*pos. The highest percentage of *FAT1*pos patients was found within the T-ALL cohort (54%, Supplementary Figure S2).

FAT1 expression was correlated with a more mature leukemic immunophenotype. In BCP-ALL, patients with a preB-ALL or a common ALL immunophenotype were in 57% and 26% classified as *FAT1*pos compared with only 9% of pro-B-ALL patients (see Supplementary Table S2). In T-ALL, a genotype–phenotype association was even more striking: 74% of patients with thymic T-ALL were *FAT1*pos compared with 45% of patients with mature T-ALL and only 4% of early T-ALL patients (see Supplementary Table S1). In accordance with the predominance of *FAT1* expression in more mature T-ALL, *FAT1*pos patients had a higher rate of clonal T-cell receptor rearrangement and a lower expression of the stem cell-associated genes *IGFBP7*, *BAALC* and *MN1* (Supplementary Table S1). Likewise, *FAT1*pos T-ALL patients showed higher white blood cell counts (WBC; $\geq 30\,000/\mu l$ at diagnosis) compared with *FAT1*neg T-ALL patients (78% vs 42%, $P<0.01$, Supplementary Table S1). No significant differences were observed between the *FAT1*pos versus *FAT1*neg groups in age or sex among T-ALL patients.

Regarding response to a standard induction therapy, we found no differences between *FAT1*pos and *FAT1*neg BCP-ALL patients (Supplementary Table S2). In T-ALL, *FAT1*neg patients failed to achieve a complete remission more frequently (3/25) after induction therapy compared with *FAT1*pos patients (0/50, $P=0.04$, Supplementary Table S1). In contrast to the *in silico* data of pediatric patients,[5] we found no differences in BCP-ALL or T-ALL between *FAT1*pos and *FAT1*neg patients regarding overall survival and remission duration (Figure 1). However, in the prognostic favorable subgroup of thymic T-ALL, we observed an inferior overall survival for *FAT1*pos patients, although not statistically significant.

On the basis of the high frequency of *FAT1* expression in T-ALL and recurrent *FAT1* mutations in early T-cell precursor (ETP)-ALL,[7] we examined 68 T-ALL samples for the presence of *FAT1* mutations by target enrichment and NGS (Supplementary Methods). Interestingly, *FAT1* mutations were detectable in a considerable number of adult T-ALL patients (8/68, 12%, Supplementary Table S3). One patient carried two point mutations within *FAT1*. All mutations were missense mutations, one leading to a frameshift and another encoding a stop codon (Supplementary Table S3). Mutations were predominantly located within the cadherin domains (Figure 2). T-ALL patients with *FAT1* mutations (*FAT1*mut) did not differ from T-ALL patients carrying a wild-type *FAT1* (*FAT1*wt) regarding sex, age, WBC and expression of specific cell surface antigens associated with an early differentiation stage. *FAT1* mutations were present in early T-ALL (3/12, 25%) and in thymic T-ALL (5/41, 12%), but absent in T-ALL with a mature immunophenotype (0/15, NS, Supplementary Table S4). Expression of *FAT1*, was more common in *FAT1*wt T-ALL patients than in *FAT1*mut patients (55% vs 25%, $P=0.15$). No differences were observed in overall survival and remission duration between *FAT1*mut and *FAT1*wt patients (Supplementary Figure S3).

Although there are increasing data on the genetic characterization of ALL, only few molecular markers have been integrated into risk stratification for individualized therapies. The postulated correlation of high *FAT1* expression with inferior outcome in

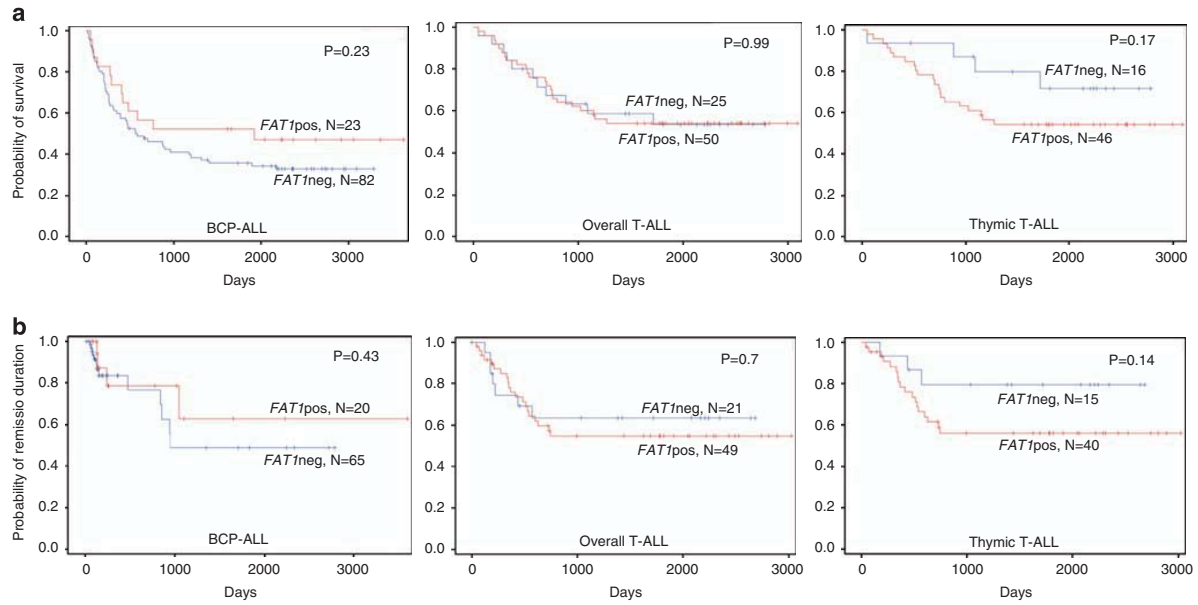

Figure 1. Overall survival (**a**) and duration of remission (**b**) for patients with BCP-ALL, overall T-ALL and the standard-risk subgroup of thymic T-ALL enrolled into GMALL trials.

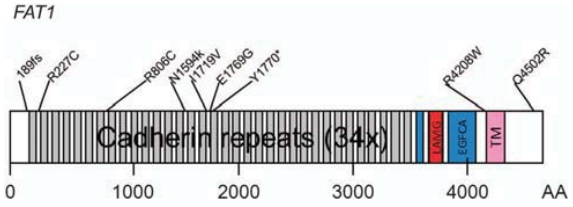

Figure 2. Protein domain plot of FAT1 with mutations ($n = 9$) found in 8 of 68 T-ALL patients. One patient carried two mutations. Changes are annotated in Supplementary Table S3.

pediatric BCP-ALL[5] could not be confirmed in our cohort of adult BCP-ALL. The most obvious reasons for these conflicting results might be different therapeutic approaches and large age differences between pediatric and adult patients as shown for other prognostic markers.[10] Also limitations of *in silico* analyses of cohorts including low number of patients might at least in part explain these conflicting findings. Although *FAT1* might not have a prognostic value, its expression and mutation profile make it an interesting candidate for minimal residual disease monitoring, the development of targeted therapies, and improved understanding of leukemogenesis in different ALL subgroups.

In addition to its potential role in leukemogenesis, it is tempting to speculate about the role of *FAT1* in the interaction of leukemic cells with the microenvironment. It is known that, *FAT1* is associated with cell migration, polarity and cell–cell adhesion and direct interaction with β-catenin.[2,4,11] As we found a high *FAT1* expression in BMSC, *FAT1* might have a role in the stabilization of the interaction of leukemic cells with the bone marrow niche and/or thymic homing. This might also explain the significantly higher expression of *FAT1* in the more differentiated subgroups of T-ALL and BCP-ALL. On the other hand, inactivating mutations of *FAT1* in different human cancers have been linked to the inability to bind β-catenin and deregulated activation of the WNT pathway.[2] These mechanisms might have a role in solid cancer leading to higher treatment sensitivity and evasion of tumor metastasis.[2,12] Interestingly, in gingiva-buccal oral squamous cell cancer, *FAT1* mutations occur in addition to mutations in *NOTCH1* and *MLL2*, a spectrum very similar to the one observed in ETP-ALL.[7,12] Deregulation of the WNT pathway has been linked to leukemogenesis in T-ALL.[13,14] The previously unreported *FAT1* mutation rate of 15% in adult T-ALL stresses the importance of the WNT pathway in T-ALL.

In conclusion, we explored the pattern of *FAT1* expression and its mutation status in a large, homogenously treated cohort of adult ALL patients. Our analysis revealed an aberrant expression predominantly in mature BCP-ALL and thymic T-ALL and a high rate of *FAT1* mutations in T-ALL. Further studies should explore a link to WNT pathway activation and potential therapeutic implications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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M Neumann[1], M Seehawer[1], C Schlee[1], S Vosberg[2,3], S Heesch[1], EK von der Heide[1], A Graf[4], S Krebs[4], H Blum[4], N Gökbuget[5], S Schwartz[1], D Hoelzer[5], PA Greif[2,3,6] and CD Baldus[1]
[1]Department of Hematology and Oncology, Charité, University Hospital Berlin, Campus Benjamin Franklin,, Berlin, Germany;
[2]Clinical Cooperative Group 'Leukemia', Helmholtz Zentrum München, German Research Centre for Environmental Health, Munich, Germany;
[3]Department of Internal Medicine 3, Ludwig-Maximilians-University, Munich, Germany;
[4]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, Ludwig-Maximilians-University, Munich, Germany;

[5]Department of Medicine II, Hematology and Oncology, Goethe University Hospital, Frankfurt, Germany and
[6]German Cancer Consortium (DKTK) and German Cancer Research Centre (DKFZ), Heidelberg, Germany
E-mail: claudia.baldus@charite.de

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6.3 *TEIL III: Identifizierung therapeutischer Ziele durch Hochdurchsatzverfahren*

Die Verfügbarkeit der neuen Sequenzierungs-Hochdurchsatzverfahren ermöglichte erstmals eine genomweite unvoreingenommene Untersuchung einzelner Nukleinsäuren in verschiedenen Tumorentitäten. Hierdurch erweiterte sich das Wissen über genetische Alterationen erheblich und offenbarte eine erstaunliche Heterogenität, sowohl zwischen verschiedenen Tumorentitäten als auch innerhalb einer Tumorentität. Wir nutzten diese Techniken, um das Spektrum genetischer Alterationen in der T-ALL des Erwachsenen offenzulegen. Neben dem Erkenntnisgewinn über grundlegende Pathomechanismen in der akuten Leukämie stand hierbei die Frage nach therapeutisch nutzbaren Veränderungen im Mittelpunkt.

6.3.1 ***Exomsequenzierung in der ETP-ALL***

(Neumann M, Heesch S, Schlee C, Schwartz S, Gökbuget N, Hoelzer D, Konstandin NP, Ksienzyk B, Vosberg S, Graf A, Krebs S, Blum H, Raff T, Brüggemann M, Hofmann WK, Hecht J, Bohlander SK, Greif PA, Baldus CD. Whole-exome sequencing in adult ETP-ALL reveals a high rate of DNMT3A mutations. Blood 2013;121:4749-4752.)

Zur molekularen Charakterisierung der Hochrisikogruppe der ETP-ALL führten wir eine Sequenzierung der proteinkodierenden DNA-Sequenzen von fünf erwachsenen ETP-ALL-Patienten durch. Als Keimbahnkontrolle wurde eine Knochenmarkprobe in kompletter Remission (MRD<10^{-3}) verwendet. Unter den insgesamt 63 identifizierten somatischen Mutationen fanden sich rekurrente Mutationen für die DNA-Methyltransferase *DNMT3A* als auch für das Cadherin *FAT3*, zusätzlich Mutationen in weiteren epigenetischen Regulatoren wie *BMI1* oder *MLL2*. Basierend auf dieser Beobachtung wurde eine größere Kohorte von insgesamt 68 ETP-ALL-Patienten mittels Sanger-Sequenzierung auf Mutationen in epigenetischen Regulatoren untersucht. *DNMT3A*, zuvor vorwiegend in der AML beschrieben, war dabei in 16% der Fälle mutiert. Ähnlich wie in der AML waren dabei vorwiegend ältere Patienten betroffen. Auch die Verteilung der Mutationen ähnelte dem von AML-Patienten mit einer häufigen Affektion der Aminosäure R882. Allerdings fand sich im Gegensatz zur AML keine Assoziation mit schlechterem Überleben. Weiterhin fanden sich *DNM2*, *FAT1*, *FAT3* und *MLL2* regelmäßig in der ETP-ALL alteriert.

Brief Report

LYMPHOID NEOPLASIA

Whole-exome sequencing in adult ETP-ALL reveals a high rate of *DNMT3A* mutations

Martin Neumann,[1] Sandra Heesch,[1] Cornelia Schlee,[1] Stefan Schwartz,[1] Nicola Gökbuget,[2] Dieter Hoelzer,[2] Nikola P. Konstandin,[3,4] Bianka Ksienzyk,[3,4] Sebastian Vosberg,[3,4] Alexander Graf,[5] Stefan Krebs,[5] Helmut Blum,[5] Thorsten Raff,[6] Monika Brüggemann,[6] Wolf-Karsten Hofmann,[7] Jochen Hecht,[8] Stefan K. Bohlander,[3,4,9] Philipp A. Greif,[3,4,10,11] and Claudia D. Baldus[1]

[1]Department of Hematology and Oncology, Campus Benjamin Franklin, Charité, University Hospital Berlin, Berlin, Germany; [2]Department of Medicine II, Hematology/Oncology, Goethe University Hospital, Frankfurt/Main, Germany; [3]Clinical Cooperative Group Leukemia, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany; [4]Department of Internal Medicine 3, and [5]Laboratory for Functional Genome Analysis, Gene-Center, Ludwig-Maximilians-University, Munich, Germany; [6]Department of Hematology and Oncology, University Hospital Kiel, Kiel, Germany; [7]Department of Hematology and Oncology, University Hospital Mannheim, Mannheim, Germany; [8]Berlin-Brandenburg Center for Regenerative Therapies, Charité Universitätsmedizin Berlin, Berlin, Germany; [9]Center for Human Genetics, Philipps University, Marburg, Germany; [10]German Cancer Consortium, Heidelberg, Germany; and [11]German Cancer Research Centre, Heidelberg, Germany

Key Points

- Exome sequencing of adult ETP-ALL reveals new recurrent mutations; in particular, *DNMT3A* is frequently mutated in adult ETP-ALL.
- More than 60% of all adult patients with ETP-ALL harbor a mutation that could potentially be targeted by a specific therapy.

Early T-cell precursor (ETP) acute lymphoblastic leukemia (ALL) is a high-risk subgroup of T-lineage ALL characterized by specific stem cell and myeloid features. In adult ETP-ALL, no comprehensive studies on the genetic background have been performed to elucidate molecular lesions of this distinct subgroup. We performed whole-exome sequencing of 5 paired ETP-ALL samples. In addition to mutations in genes known to be involved in leukemogenesis (*ETV6*, *NOTCH1*, *JAK1*, and *NF1*), we identified novel recurrent mutations in *FAT1* (25%), *FAT3* (20%), *DNM2* (35%), and genes associated with epigenetic regulation (*MLL2*, *BMI1*, and *DNMT3A*). Importantly, we verified the high rate of *DNMT3A* mutations (16%) in a larger cohort of adult patients with ETP-ALL (10/68). Mutations in epigenetic regulators support clinical trials, including epigenetic-orientated therapies, for this high-risk subgroup. Interestingly, more than 60% of adult patients with ETP-ALL harbor at least a single genetic lesion in *DNMT3A*, *FLT3*, or *NOTCH1* that may allow use of targeted therapies. (*Blood*. 2013;121(23):4749-4752)

Introduction

T-lineage acute lymphoblastic leukemia (T-ALL) accounts for approximately 25% of all adult ALL. The novel high-risk subgroup of early T-cell precursor (ETP) represents 10% of all adult T-ALL. This subgroup is characterized by a specific immunophenotype (CD1-, $CD5_{weak}$, CD8-, or coexpression of stem cell and/or myeloid markers) and distinct molecular features (*FLT3* mutations and an absence of *NOTCH1* mutations).[1,2] ETP-ALL, regarded as stem cell–like leukemia, is associated with a high risk for treatment failure for both pediatric and adult patients. Because of the myeloid features of ETP-ALL, it was proposed that these patients might benefit from therapies used in myeloid malignancies, including high-dose cytarabine or epigenetic modulators.[3]

Although in pediatric ETP-ALL whole-genome sequencing has revealed a highly heterogeneous picture of genetic alterations,[4] no comprehensive molecular analyses have been performed in adult ETP-ALL. Here we systematically screened for somatic mutations in adult ETP-ALL. We have analyzed exomes of 5 patients and subsequently verified mutations in *DNMT3A* and members of the polycomb repressor complex 2 (PRC2) in a larger cohort of adult patients with ETP-ALL.

Study design

Patients

We investigated paired bone marrow samples of 5 adult patients with ETP-ALL (supplementary Methods and supplementary Table 1, available on the Blood Web site) with sufficient genomic DNA at diagnosis and in remission (minimal residual disease[5] level $< 4 \times 10^{-3}$) that had been sent to the reference laboratory within the German Acute Lymphoblastic Leukemia Multicenter (GMALL) study group. In addition, 68 adult patients with ETP-ALL (55 men, 13 women; median age, 38 years) were analyzed for the mutation status of selected genes (*DNMT3A*, *EZH2*, *EP300*, *SH2B3*, and *SUZ12*). For 52 (76%) of the 68 patients, clinical follow-up data were

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P.A.G. and C.D.B. contributed equally to this study.

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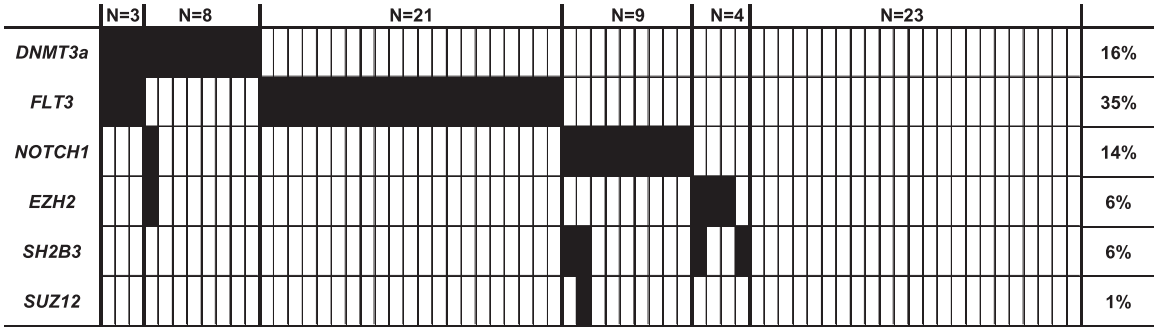

Figure 1. The spectrum of mutations in *DNMT3A*, *FLT3*, *NOTCH1*, *EZH2*, *SH2B3*, and *SUZ12* in 68 adult patients with ETP-ALL.

available (supplementary Tables 2 and 3). All patients gave written informed consent to participate in the study according to the Declaration of Helsinki. The studies were approved by the ethics board of the Johann Wolfgang Goethe-Universität of Frankfurt/Main in Germany.

Exome sequencing and mutation analysis

The methods are described in the supplemental Data. In brief, exome sequencing was performed for 5 adult patients with ETP-ALL, as previously reported,[6,7] using exon capturing from genomic DNA (Sure Select target enrichment; Agilent, Santa Clara, CA), followed by 76-bp paired-end sequencing on an Illumina Genome Analyzer IIx platform (Illumina, San Diego, CA). Candidate variants were confirmed by capillary sequencing of genomic DNA.

Mutation analyses for *EZH2* (exons 14-20), *SH2B3* (exons 2-8), *DNMT3A* (exons 13-23), *EP300* (exon 30), and *SUZ12* (exons 14-15) were performed by Sanger sequencing in 68 adult patients with ETP-ALL. Primers are listed in the supplement (supplementary Table 4). All samples had been previously analyzed for *FLT3* and *NOTCH1* mutations.[2]

Statistical analyses were performed using SPSS software version 17 (SPSS Inc, Chicago, IL) and GraphPad Prism software version 5 (GraphPad Software Inc, La Jolla, CA).

Results and discussion

As no comprehensive analysis exists on the molecular background of the high-risk subgroup of adult ETP-ALL, we performed whole-exome sequencing in 5 adult patients (supplementary Table 1). At least 3.8 Gbp of exome sequence was generated for each sample (supplementary Table 5). We found a total of 63 nonsynonymous somatic mutations or indels in 5 patients with ETP-ALL (range, 6-16 per patient). Eleven mutations/indels affected cancer genes, according to the census of human cancer genes[8] (see also http://www.sanger.ac.uk/genetics/CGP/Census). Recurrent mutations affected *DNMT3A* and *FAT3* (*FAT*, tumor suppressor homolog 3) in 2 of the 5 patients. The DNA-methyl-transferase *DNMT3A* is a frequent mutational target in acute myeloid leukemia (AML; 20%),[9] whereas *FAT3* mutations were reported in ovarian carcinoma.[10] New mutational targets identified involved genes in epigenetic regulation (*MLL2*, *BMI1*, and *FAT1*) and genes reported to be mutated in pediatric ETP-ALL and other ALL subgroups (*JAK1*, *ETV6*, *NOTCH1*, *DNM2*, and *NF1*)[4,11,12] (supplementary Table 6).

Deregulated DNA methylation is a commonly found in many malignancies.[13] *DNMT3A* is mutated in myelodysplastic syndrome (8%) and AML,[9,14] where it is associated with a poor prognosis.[9,15-18] As the same mutations occur in myelodysplastic syndrome and the corresponding secondary AML,[19] *DNMT3A* mutations likely represent an early event in leukemogenesis.[20] Until now, a single study has reported a single *DNMT3A* mutation in T-ALL, and just recently, a second paper described frequent mutations of *DNMT3A* in adult T-ALL, although not specifying ETP-ALL.[21,22] Interestingly, no *DNMT3A* mutations were identified by whole-genome sequencing in pediatric ETP-ALL.[4]

By Sanger sequencing, we determined the frequency of *DNMT3A* mutations in a large cohort of adult ETP-ALL. In 16% of the 68 patients with ETP-ALL, *DNMT3A* was mutated, reflecting a mutation rate similar to that of AML, with a similar mutation pattern and mutational hot spot in exon 23. Amino acid R882 of *DNMT3A* was mutated in 5 ETP-ALL samples. Four of the 6 remaining mutations were previously described in AML (supplementary Figure 1).[9,15,23]

With respect to clinical characteristics, no difference was observed between *DNMT3A* mutated and wild-type patients regarding sex distribution. In addition, *DNMT3A* mutated and wild-type patients did not differ in the immunophenotype or the mutational status of *NOTCH1* and *FLT3* (Table 1 and supplementary Table 3).

Table 1. Characteristics of adult patients with ETP-ALL and comparison between *DNMT3A* mutated and *DNMT3A* wild-type patients

	DNMT3mut (n = 11)		*DNMT3wt* (n = 57)		
Characteristic	**n**	**%**	**n**	**%**	***P***
Sex					.35
Female	3	27.3	10	17.6	
Male	8	72.7	47	82.4	
Age, y					.02
Median	63		37		
Range	24-73		17-74		
Clinical data available					.27
n	7	64	45	79	
FLT3					.41
Mutated	3	27.3	21	36.8	
Wild-type	8	72.7	36	63.2	
NOTCH1					.68
Mutated	1	9.1	9	15.8	
Wild-type	10	90.9	48	84.2	
EZH2					.52
Mutated	1	9.1	3	5.3	
Wild-type	10	90.9	54	94.7	
SH2B3					.49
Mutated	0	0	4	7	
Wild-type	11	100	53	93	
SUZ12					.84
Mutated	0	0	1	1.8	
Wild-type	11	100	56	98.2	

Thus, in contrast to AML, no association between *DNMT3A* and *FLT3* mutations can be found.[9,15,23] Similar to in AML,[9,16] patients with ETP-ALL with *DNMT3A* mutations were significantly older (median, 63 vs 37 years; $P = .016$) than patients with *DNMT3A* wild-type (Table 1). The lack of *DNMT3A* mutations in pediatric ETP-ALL and the low rate of *DNMT3A* mutations in pediatric AML[24,25] point toward age-specific genetic differences. Because of the occurrence of genetic alterations in epigenetic regulators, we verified genetic mutations, including members of PRC2 in a larger ETP-ALL cohort (n = 68). Mutations were found for *EZH2* in 4 of 68 (6%) and *SUZ12* in 1 of 68 (1%) of adult ETP-ALL (supplementary Figure 2). No mutation was found in *EP300*. Thus, in contrast to pediatric ETP-ALL, with a high rate of mutations in PRC2,[4,21] we found a low rate of PRC mutations in adult ETP-ALL. *DNMT3A* mutations had no significant prognostic effect in this small cohort of adult ETP-ALL. Patients with at least a single mutation in epigenetic regulators (*DNMT3A*, *SUZ12*, *EP300*, or *EZH2*) showed a trend toward inferior survival compared with unaffected patients (1-year survival, 56% vs 88%; log rank, P value = .24; Breslow, P value = .16; supplementary Figure 3).

To further explore these genetic alterations as recurrent events, additional patients with ETP-ALL (n = 20) were analyzed by targeted next-generation sequencing (supplemental Data). In addition to *DNMT3A*, mutations in *MLL2* (10%), previously linked to lymphoma,[26] underscore the relevance of alterations in epigenetic regulators. Moreover, mutations in the cadherins *FAT1* (25%) and *FAT3* (20%) implicate alterations in cell adhesion, interaction mechanisms, and activation of the Wnt pathway.[27] In particular, *FAT1* may act as a tumor suppressor.[27] Finally mutations *DNM2* (35%) and *JAK1* (15%)[4] point to molecular targets for novel therapies (supplementary Figure 4, supplementary Figure 5, and supplementary Table 7).

In conclusion, adult ETP-ALL displays a distinct mutation spectrum, particularly affecting genes involved in epigenetic regulation (in particular, *MLL2*, *DNMT3A*, and *PRC2*). This mutation spectrum is different for pediatric patients, with a lower rate of PRC2 mutations and a higher rate of *DNMT3A* mutations pointing toward distinct molecular alterations in adult ETP-ALL. This might result from preexisting lesions in the hematopoietic progenitors, as it was shown that *TET2* mutations result in an age-related myeloid lineage bias in elderly individuals.[28] *DNMT3A* mutations in adult ETP-ALL show a similar frequency to that seen in AML, are located at the same hot spot, and are in conjunction with mutations in other epigenetic regulators of potential unfavorable prognostic effect, underscoring the stem cell/myeloid origin of ETP-ALL. These data may provide a potential rationale to study epigenetic directed therapies in the high-risk subgroup of ETP-ALL. Importantly, we can now define at least 3 different molecular subgroups of adult ETP-ALL characterized by alterations in epigenetic regulators (25%), mutations in *FLT3* (35%) and other kinases (*JAK* pathway), and the presence of *NOTCH1* mutations (15%) (Figure 1).[2] In total, more than 60% of all adult patients with ETP-ALL harbor alterations that can potentially be molecularly targeted by new treatment approaches such as demethylating agents, kinase inhibitors, and γ secretase inhibitors.

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Authorship

Contribution: M.N. contributed to the research, analyzed data, and wrote the manuscript; S.S. supervised the reference laboratory and reviewed the manuscript; N.G. supervised the GMALL study center and reviewed the manuscript; D.H. supervised the GMALL study group; S.H., C.S., N.P.K., B.K., S.V., A.G., S.K., H.B., T.R., M.B., J.H., and S.K.B. contributed to the research; W.-K.H. contributed to the study analysis and reviewed the manuscript; P.A.G. designed the research, contributed to the data analysis, and edited the manuscript; C.D.B. designed the research, contributed to the data analysis, and edited the manuscript; and P.A.G. and C.D.B. jointly supervised the study.

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Correspondence: Claudia D. Baldus, Charité, University Hospital Berlin, Campus Benjamin Franklin, Department of Hematology and Oncology, Hindenburgdamm 30, 12203 Berlin, Germany; e-mail: claudia.baldus@charite.de.

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6.3.2 ***Charakterisierung des Mutationsspektrums der erwachsenen T-ALL***

(**Neumann M**, Vosberg S, Schlee C, Heesch S, Schwartz S, Gökbuget N, Hoelzer D, Graf A, Krebs S, Bartram I, Blum H, Brüggemann M, Hecht J, Bohlander SK, Greif PA, Baldus CD. Mutational spectrum of adult T-ALL. Oncotarget. 2015 Feb 20;6(5):2754-66)

Um das Mutationsspektrum der erwachsenen T-ALL zu charakterisieren, führten wir Untersuchungen der kompletten proteinkodierenden Regionen von 88 Genen in 81 T-ALL-Patienten durch. Die Genauswahl erfolgte literaturbasiert auf dem Boden der berichteten alterierten Gene in der T-ALL, aber auch der BCP-ALL und AML Zusätzlich wurden in das „bait-basierte“ Genpanel (Agilent SureSelect) Gene von regelmäßig betroffen Signalwegen (NOTCH-Pathway, JAK/STAT-Pathway, epigenetische Regulatoren) aufgenommen. Die Sequenzierung der zielregionangereicherten Bibliotheken wurde auf einem Illumina HiSeq vorgenommen. Die durchschnittliche Abdeckung von 130-facher Lesetiefe pro Nukleinsäure erlaubte auch die Detektion von subklonalen Strukturen. Insgesamt waren insgesamt 66 der 88 Gene mindestens in einem Patienten verändert. 15 Gene zeigte eine Mutationsfrequenz von mehr als fünf Prozent, acht Gene waren in mehr als 10% der Patienten mutiert. Bekannte Mutationsfrequenzen konnten validiert werden (u.a. für *NOTCH1*, *WT1*, *FBXW7*, *BCL11B*), für die erwachsene T-ALL erstmals in einer größeren Kohorte beschrieben werden (u.a. *DNM2*, *MLL2*, *FAT1*, *FAT3*) oder bei bislang divergierenden Angaben in einer einheitlichen Kohorte determiniert werden (*JAK1*, *JAK3*). Zusätzlich fanden sich neue rekurrente Alterationen (*HERC1*, *NOTCH2*, *ZRSF2*).

Neben dem NOTCH-Signalweg (>70%) waren vor allem epigenetische Regulatoren (33%) und der JAK/STAT-Pathway (18%) betroffen. Für diese Signalwege bestehen potentielle zielgerichtete Therapien, insgesamt fanden sich in über 80% der T-ALL-Patienten alterierte Strukturen in Signalwegen, die medikamentös variierbar sind.

Die Heterogenität der molekularen Alterationen in der erwachsenen T-ALL zeigt sich nicht nur in mit Ausnahme von *NOTCH1* eher niedrigen Mutationsfrequenzen, sondern auch noch innerhalb jedes einzelnen Patienten. Es liegen meist mehrere Mutationen mit unterschiedlichen „variant allele frequencies“ (VAF) vor, die auf subklonale Strukturen hinweisen und mit dem Auftreten von Rezidiven und Therapieresistenz in Verbindung gebracht werden können. Exemplarisch sei hierfür *NOTCH1* genannt, welches zwar häufig eine VAF zwischen 40 und 50% aufweist und damit wahrscheinlich dem Hauptklon zuzuordnen ist, aber in einigen Patienten auch mit niedrigeren Frequenzen und damit subklonal auftritt. Zusätzlich gibt es Patienten mit gleichzeitig mehreren, spekulativ biallelischen, Mutationen von *NOTCH1*.

Inwieweit Patienten mit subklonalen Strukturen von einer zielgerichteten Therapien profitieren, bleibt weiteren Studien vorbehalten.
Insgesamt bildet die Beschreibung des molekularen Spektrums der T-ALL eine Basis für die Implementierung von zielgerichteten Therapien.

Mutational spectrum of adult T-ALL

Martin Neumann[1], Sebastian Vosberg[2,3], Cornelia Schlee[1], Sandra Heesch[1], Stefan Schwartz[1], Nicola Gökbuget[4], Dieter Hoelzer[4], Alexander Graf[5], Stefan Krebs[5], Isabelle Bartram[1], Helmut Blum[5], Monika Brüggemann[6], Jochen Hecht[7], Stefan K. Bohlander[2,8], Philipp A. Greif[2,3,9,10,*] and Claudia D. Baldus[1,9,*]

[1] Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Department of Hematology and Oncology, Berlin, Germany

[2] Clinical Cooperative Group 'Leukemia', Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany

[3] Department of Internal Medicine 3, Ludwig-Maximilians-Universität (LMU), Munich, Germany

[4] Goethe University Hospital, Department of Medicine II, Hematology/Oncology, Frankfurt/M., Germany

[5] Laboratory for Functional Genome Analysis, Gene-Center, Ludwig-Maximilians-Universität (LMU), Munich, Germany

[6] University Hospital Kiel, Department of Hematology, University Hospital Schleswig-Holstein, Campus Kiel, Germany

[7] Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité Universitätsmedizin Berlin, Berlin, Germany

[8] Department of Molecular Medicine and Pathology, The University of Auckland, Auckland, New Zealand

[9] German Cancer Consortium (DKTK), Heidelberg, Germany

[10] German Cancer Research Centre (DKFZ), Heidelberg, Germany

[*] These authors contributed equally to this work

***Correspondence to**: Claudia D. Baldus, **email**: claudia.baldus@charite.de*

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ABSTRACT

Novel target discovery is warranted to improve treatment in adult T-cell acute lymphoblastic leukemia (T-ALL) patients. We provide a comprehensive study on mutations to enhance the understanding of therapeutic targets and studied 81 adult T-ALL patients. *NOTCH1* exhibited the highest mutation rate (53%). Mutation frequencies of *FBXW7* (10%), *WT1* (10%), *JAK3* (12%), *PHF6* (11%), and *BCL11B* (10%) were in line with previous reports. We identified recurrent alterations in transcription factors DNM2, and RELN, the WNT pathway associated cadherin *FAT1*, and in epigenetic regulators (*MLL2*, *EZH2*). Interestingly, we discovered novel recurrent mutations in the DNA repair complex member *HERC1*, in *NOTCH2*, and in the splicing factor *ZRSR2*. A frequently affected pathway was the JAK/STAT pathway (18%) and a significant proportion of T-ALL patients harboured mutations in epigenetic regulators (33%), both predominantly found in the unfavourable subgroup of early T-ALL. Importantly, adult T-ALL patients not only showed a highly heterogeneous mutational spectrum, but also variable subclonal allele frequencies implicated in therapy resistance and evolution of relapse. In conclusion, we provide novel insights in genetic alterations of signalling pathways (e.g. druggable by γ-secretase inhibitors, JAK inhibitors or EZH2 inhibitors), present in over 80% of all adult T-ALL patients, that could guide novel therapeutic approaches.

INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) in adults represents a disease with an unfavorable outcome[1]. While the cure rate in pediatric T-ALL patients exceeds 70%, a similar rate in adults is only observed for patients in the favorable risk group of thymic T-ALL as defined by the expression of CD1a[2,3].

Patients within the immature immunophenotypic group of early T-ALL as well as patients of the mature T-ALL subtype show a significantly inferior outcome[1]. Although allogeneic stem cell transplantation (alloSCT) in first complete remission has led to an improved outcome e.g. in the context of the German Multicenter Acute Lymphoblastic Leukemia (GMALL) trials, further therapeutic improvements are urgently warranted, in particular for high risk patients. In B-cell precursor (BCP-) ALL molecularly directed therapies like Rituxumab[4], the bi-specific antibody Blinatumomab[5], or tyrosine kinase inhibitors in Ph+-ALL[6] are well established. In contrast, targeted therapies are not available for T-ALL, with the exception of Nelarabine[7]. In order to identify targets for specific treatment strategies, a better understanding of the molecular background of T-ALL is necessary[8].

Previous to next generation sequencing (NGS), genetic alterations of leukemic blasts were mainly examined by cytogenetics to detect chromosomal rearrangements. In addition, immunophenotypic characterization, gene expression arrays, and copy number alterations were also used to categorize T-ALL[9]. Variations on a single nucleotide level had only been described for very few genes. One of the most relevant and frequent alterations are mutations of *NOTCH1* gene occurring in about 60% of all T-ALL cases[10-12]. In addition, mutations of *FBXW7*, another player in the NOTCH pathway, as well as mutations of *WT1* and *PTEN* were previously described[13-18].

Through NGS, molecular classification of T-ALL has dramatically expanded. Recurrent mutations in T-ALL affect genes involved in transcriptional processes (*BCL11B*[19], *RUNX1*[20], *GATA3*[21]), epigenetic regulation (*DNMT3A*[20,22], members of polycomb repressor complex (PRC2)[21]), JAK/STAT signalling[21,23-25] (*JAK1*/2/3, *IL7R*), ribosomal processes (*RPL10, RPL5)*[25], and various other functions (e.g. *WT1*[26], *CNOT3*[25], *PHF*[27], *MEF2C*[28,29], *LEF1*[30]). These data, predominantly derived from pediatric T-ALL, suggest a highly heterogeneous and complex molecular background of T-ALL. While some of these alterations imply prognostic significance, comprehensive studies with focus on therapeutic targets in larger series of adult T-ALL patients are missing. This is of particular importance as most cancer genes occur at intermediate frequencies of 2-20% or even lower[31].

Thus far, molecular subgroups in T-ALL were defined mainly based on gene expression profile (GEP)[28,32,33] or immunophenotype[2,3]. Both classifications, based on GEP or immunophenotype reflect the physiological T-cell stage, in which growth arrest and malignant transformation occured[34]. The gene signature of the early T-cell precursor (ETP)-ALL reflects the expression profile of early thymocyte progenitors in the double negative (DN)1 stage and ETP-ALL also shows a distinct immunophenotype[35,36]. Importantly, ETP-ALL, which recently gained interest as it represents a subgroup of T-ALL with stem cell and myeloid characteristics[21,36-38], may serve as model for the design of novel molecular therapies. Although the classification of ETP-ALL based on gene expression and immunophenotype were only partly overlapping[29], the subgroup of ETP-ALL is already an ideal model for therapy approaches adapted to its distinct molecular characteristics. Specifically mutated genes, mainly affected in ETP-ALL include members of the (PRC2) or genes reflecting the stem cell and myeloid character of ETP-ALL like *FLT3, DNMT3A* or *KRAS*. This mutation pattern of ETP-ALL opens up potential options for targeted therapies[21,22]. This might be of special interest in a minimal residual disease (MRD) setting as a bridging therapy to alloSCT.

Whereas a number of putative driver mutations have been characterized, the spectrum of recurring alterations in larger cohorts and their relevance in different T-ALL subgroups remains unexplored. To unravel this spectrum and to explore potential targets for novel therapeutic interventions, we performed targeted high throughput sequencing of 88 candidate genes in 81 T-ALL samples of adult patients.

RESULTS

Single nucleotide variations and short indels in adult T-ALL

We obtained an average of 1.2 million reads for each sample resulting in an average coverage of 120 reads for the target region. Eighty percent of the targeted region was covered with a minimum of 20 reads (Supplementary Table S1). After exclusion of polymorphisms annotated in dbSNP135, 473 single nucleotide variations (SNVs) and short indels were identified with a minimum call of 20 reads, 313 of those resulted in changes in the coding sequence of the target region. On average, three (3.2) genes per patient were mutated, and 64 (73%) of the 88 genes were mutated in at least one patient (Supplementary Table S2). We identified three patients without any SNVs in the selected genes. One patient showed an aberrantly high rate of SNVs with 21/88 genes being mutated (Supplementary Table S3). The number of mutations in the selected genes did not correlate with the patients' age.

Mutational spectrum of candidate genes in T-ALL

In total, fifteen of the 88 investigated genes were mutated in more than 5% of patients with nine genes showing a mutation frequency of ≥10%. As expected, the highest mutation rate with 53% was found for *NOTCH1*. Mutation frequencies of *FBXW7* (10%), *WT1* (10%), *JAK3*

Table 1: Mutational spectrum and comparison of T-ALL subgroups. Genes with mutations detected in at least 3% of the examined samples are shown. In parentheses are the percentages for each subgroup

	Mutational spectrum			
		T-ALL subgroups		
	total	thymic	mature	early
n	81	40	15	26
NOTCH1	43 (53.1%)	27 (67.5%)	6 (40.0%)	10 (38.4%)
DNM2	14 (17.3%)	7 (17.5%)	2 (13.3%)	5 (19.2%)
FAT1	13 (16.0%)	6 (15.0%)	1 (6.7%)	6 (23.0%)
FAT3	11 (13.6%)	5 (12.5%)	2 (13.3%)	4 (15.3%)
JAK3	11 (13.6%)	3 (7.5%)	3 (20.0%)	5 (19.2%)
PHF6	11 (13.6%)	5 (12.5%)	1 (6.7%)	5 (19.2%)
MLL2	10 (12.3%)	5 (12.5%)	1 (6.7%)	4 (15.3%)
FBXW7	9 (11.1%)	9 (22.5%)	0 (0.0%)	0 (0.0%)
WT1	8 (9.9%)	4 (10.0%)	0 (0.0%)	4 (15.3%)
BCL11B	7 (8.6%)	5 (12.5%)	2 (13.3%)	0 (0.0%)
HERC1	7 (8.6%)	4 (10.0%)	2 (13.3%)	1 (3.8%)
RELN	7 (8.6%)	5 (12.5%)	1 (6.7%)	1 (3.8%)
RUNX1	7 (8.6%)	1 (2.5%)	2 (13.3%)	4 (15.3%)
PTEN	6 (7.4%)	4 (10.0%)	1 (6.7%)	1 (3.8%)
DNMT3A	5 (6.2%)	1 (2.5%)	1 (6.7%)	3 (11.5%)
CBL	4 (4.9%)	1 (2.5%)	2 (13.3%)	1 (3.8%)
EP300	4 (4.9%)	2 (5.0%)	1 (6.7%)	1 (3.8%)
JAK1	4 (4.9%)	1 (2.5%)	1 (6.7%)	2 (7.6%)
MTOR	4 (4.9%)	3 (7.5%)	1 (6.7%)	0 (0.0%)
SUZ12	4 (4.9%)	0 (0.0%)	1 (6.7%)	3 (11.5%)
TET2	4 (4.9%)	4 (10.0%)	0 (0.0%)	0 (0.0%)
WHSC1	4 (4.9%)	2 (5.0%)	0 (0.0%)	2 (7.6%)
BCOR	3 (3.7%)	3 (7.5%)	0 (0.0%)	0 (0.0%)
ETV6	3 (3.7%)	0 (0.0%)	0 (0.0%)	3 (11.5%)
MTMR3	3 (3.7%)	0 (0.0%)	2 (13.3%)	1 (3.8%)
PRKCZ	3 (3.7%)	3 (7.5%)	0 (0.0%)	0 (0.0%)
ZRSR2	3 (3.7%)	3 (7.5%)	0 (0.0%)	0 (0.0%)

(12%), and *BCL11B* (10%) were in the range of previously reported frequencies[21,26,39,40]. Recently identified recurrent alterations in *DNM2* (17%), *PHF6* (11%), *DNMT3A* (5%) or *RELN* (5%) were confirmed in this larger cohort of adult T-ALL patients[20,21,27] (Table 1). Interestingly, genes involved in epigenetic functions such as *TET2* (5%), *SUZ12* (5%), *EP300* (5%) as well as genes that possess transcriptional activity like *RUNX1* (9%), *PTEN* (8%), *CBL* (5%), or *BCOR* (4%) were mutated in the range of 5-10% of our T-ALL patients.

Genes previously linked to ETP-ALL were also found to be mutated in the remaining non-ETP T-ALL subgroups including recurring mutations in the histone methyl-transferase *MLL2* (11%), frequently mutated in B-cell lymphomas[41-43]. Like in B-cell lymphoma, *MLL2* mutations were distributed over the entire gene locus without pointing towards a hot-spot region (Supplementary Figure S1). Similarly, the protocadherins *FAT1* (15%) and *FAT3* (12%) were altered not only in early T-ALL (*FAT1* 23%, *FAT3* 15%), but were also recurrently

mutated - though in a lower frequency - in thymic T-ALL (*FAT1* 15%, *FAT3* 13%; Table 1).

Mutation frequencies for distinct genes appeared to significantly different across T-ALL subgroups (Figure 1a). This was most remarkable for members of the NOTCH pathway: *NOTCH1* showed a higher frequency in thymic (67.5%) compared to early T-ALL (38.4%, P=0.02). Consistent with this mature immunophenotype, *NOTCH1* mutation status was significantly linked to a clonal TCR rearrangement (64% clonal TCR rearrangement in *NOTCH1*mut vs. 36% in *NOTCH1*wt, P=0.01). *FBXW7* mutations, similar to *NOTCH1* mutations, occurred exclusively in the subgroup of thymic T-ALL (Table 2). Additional mutations exclusively found in the subgroup of thymic T-ALL included *BCL11B*, *TET2*, *MTOR*, *BCOR*, and *ZSRS2*. In contrast, genes of the JAK/STAT pathway (*JAK1*, *JAK3*) and the PRC2 complex (*EZH2*, *SUZ12*) as well as the transcription factors *ETV6* and *RUNX1* were predominantly mutated in the immature T-ALL subgroup (Figure 1a).

In addition, we found novel mutations in genes which, to our knowledge, have not yet been reported in T-ALL. Among these *HERC1*, functionally involved in DNA repair, was among the most frequently mutated genes. Other recurrently affected genes included the splicing gene *ZRSR2*, or *PRKCZ*, a gene also involved in

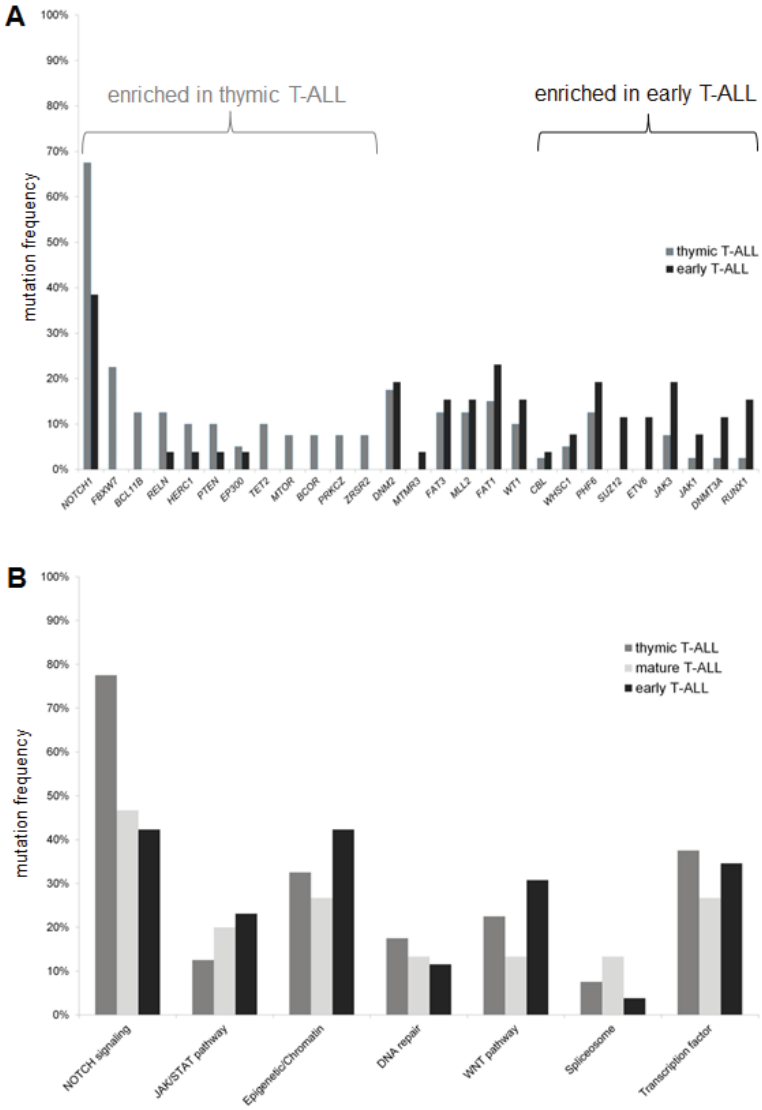

Figure 1: Comparison of mutation frequencies between the different T-ALL subgroups. (A) Distribution for single genes and (B) according to the related pathways. Only genes with a mutation rate higher than 3% are shown.

DNA repair (Supplementary Table S3).

Overall, there was no obvious association between the mutation status of different genes. Some of the genes with low mutation rates occurred exclusively, including genes with redundant functions like e.g. the histone methyltransferases, *WHSC1* and *MLL2*. *WHSC1* (also known as *NSD2* or *MMSET2*) is associated with the prognostic unfavourable t(4;14) subgroup in multiple myeloma[44] and only very recently described in T-ALL[45,46]. We found *WHSC1* to be mutated in 6% of the patients in our cohort. When combining *WHSC1* and *MLL2* mutated cases, 17% of all patients revealed alterations of histone methyltransferase genes.

Affected pathways and association with T-ALL subgroups

To address the complexity of this heterogeneous mutational spectrum, we focused on pathways with potential targets. In this study, the NOTCH pathway was affected in about 60% of all T-ALL patients (Figure 1B), including mutations in *NOTCH1* and *FBXW7* as well as in *NOTCH2*, *NOTCH3*, *HES1*, *JAG1*, and *JAG2* (Supplementary Table S3). Mutations involving the *NOTCH* pathway were predominant in the thymic subgroup (75%) as compared to the early T-ALL (33%, P=0.004) subgroup. The spectrum of additional mutations between *NOTCH1* mutated and *NOTCH1* wildtype patients was not significantly different.

Interestingly, over 35% of our T-ALL patients carried lesions in epigenetic modulators. Whereas DNA methylation modifiers (like *DNMT3A*, *TET2*, *IDH1*, *IDH2*) were affected in 9% of all cases, histone modifiers were even more frequently altered, including members of the PRC such as *SUZ12*, *EZH2*, or *EP300* and the histone methyltransferases *MLL2* and *WHSC1* (28%, Figure 2). Interestingly, chromatin modifying genes were slightly more frequently mutated in early compared to thymic T-ALL (42% vs. 32%, n.s.; Figure 1B).

The JAK/STAT pathway is of particular interest for the design of targeted therapies with the emergence of JAK inhibitors. Mutations in *JAK1*, *JAK2*, *JAK3*, *IL7R* occurred in 19% of all T-ALL patients, but these preferentially occurred in immature, high risk T-ALL cases. Among those, *JAK3* mutations were frequent (14%) and preferentially found in the early (19%) and mature (20%) subgroups compared to thymic T-ALL (8%, n.s., Table 1, Figure 1 and 2).

Another pathway of interest is the WNT pathway with a high rate of mutations in *FAT1* and *FAT3*, which is frequently altered in the immature T-ALL subgroups (Figure 2). The mutation frequency of *LEF1*, a main player in the WNT pathway, was unexpectedly low (1%), which may be due to the fact that larger deletions could be missed with our NGS approach.

Spliceosome mutations, described for myeloid and mature lymphoid malignancies, were present only in a minority (7.4%) of T-ALL (Figure 1B). Overall, pathways with a potential targeted treatment option were affected in

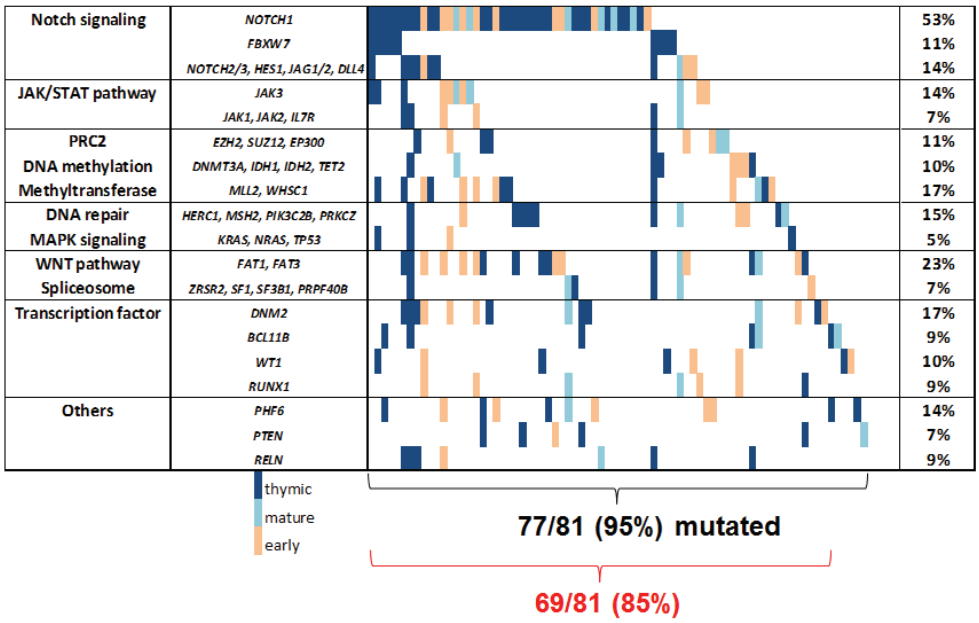

Figure 2: Mutational landscape of adult T-ALL. In the right column mutations rates are shown for groups with functional similarity. The red brackets summarize pathways representing potential therapeutic targets and their frequency. Genes with a mutation rate below 5% are grouped with functional similar genes or are not shown.

85% of all T-ALL patients. These included the NOTCH pathway, JAK/STAT pathway, WNT pathway, DNA methylation, chromatin modifying enzymes, spliceosome, and MAPK pathway (Figure 2).

Variable allele frequencies suggest subclonal mutations

To identify mutations that may originate from the founding clone, we analysed the variant allele frequencies (VAFs) of all SNVs. In our cohort, T-ALL samples showed a wide spectrum of VAFs. For a founding clone, VAFs would be expected to be 44% (+/-7%)[47]. Within this T-ALL cohort, samples differed not only in the number of mutated genes, but also in range of VAFs for targeted genes.

The number of mutated genes varied widely across different patients (0-21/patient). The group of patients (n=13) with more than five mutated genes included cases with the majority of mutated genes linked to the founding clone. However, three cases had only one gene with VAFs

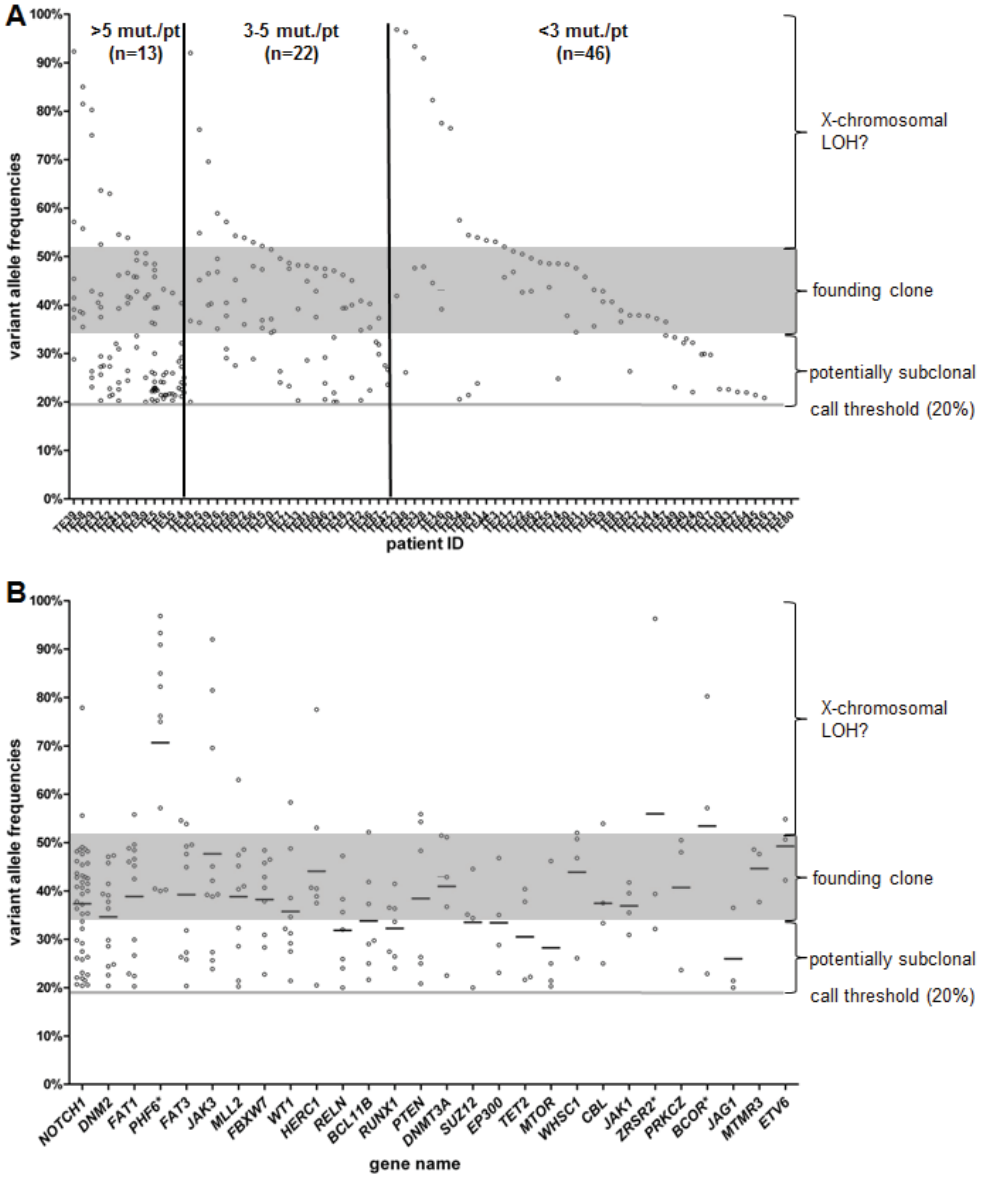

Figure 3: Variant allele frequencies (VAFs) of each individual patient (A) and each gene (B) are shown. The grey shaded zone displays the expected range for a VAF in the founding clone.

of greater than 40% and the VAFs of the remaining genes were below 30%, pointing towards a subclonal structure of the leukemia. In patients with three or more mutated genes (n=36), we found at least one gene with VAFs in the range of a founding clone. On the other hand, we found 16 (18%) samples without any alteration with a VAF in the range of a founding clone (Figure 3A). These were all patients with two or less mutated genes and it is likely that the driver mutation was missed due to the gene selection.

The ideal target for an individualized treatment approach would be a driver mutation with a VAF in a founding clone. Although some of the genes with a mutation rate over 5% had predominantly VAFs over 40%, e.g. *NOTCH1* or *FAT1*, none of those could be exclusively assigned as founding clone. Interestingly, *NOTCH1* regarded as a prominent driver in T-ALL was found mutated on a subclonal level in 16 patients (37% of all *NOTCH1* mutated patients). Furthermore, in three patients *NOTCH1* showed at least two different alterations (Supplementary Figure S1). In all these cases one mutation had a VAF in the range of a founding clone, but the second mutation appeared to be present only within a subclone with a VAF below 30%.

Most of the recurrently mutated genes could be assigned to a founding clone in at least one patient. Some genes only showed low VAFs like e.g. *ABL1*, *FLT3*, *NRAS* or *SUZ12* and thus are presumably later events in leukemogenesis. All of these genes were mutated in only three or less T-ALL patients in our cohort. Among the genes with a VAF higher than 50%, *PHF6*, *BCOR*, and *ZRSR2* are located on the X chromosome (Figure 3B). Taken together, the spectrum of VAFs in T-ALL shows a highly heterogeneous pattern with none of the frequent (>10%) lesions being exclusively present in the founding clone.

DISCUSSION

Although risk stratification and subsequent therapy intensification have led to an improved outcome in adult T-ALL, the cure rate of approximately 50% remains unsatisfactory. Unlike in BCP-ALL with established targeted therapies (Rituximab, TKI, potentially Blinatumomab), no targeted therapy is yet available in T-ALL. Therefore, molecular targets and implementation of individualized treatment options are sorely needed.

In our study, we investigated the mutational spectrum of a large adult T-ALL cohort to identify potential molecular targets. As previously reported, T-ALL shows, despite of common features regarding immunophenotype or gene expression, a highly heterogeneous mutational background[21,25]. However, most of the previously published data are generated in pediatric T-ALL patients. Here, we have investigated in an original and comprehensive study, a large set of candidate genes in a large cohort of adult T-ALL patients. This approach would allow us to identify also recurrent candidate genes altered in lower frequencies[31]. We were able to confirm a broad spectrum of mutations and this provides confidence that the detection of genetic lesions is accurately displayed in sequencing libraries and targeted NGS.

Among the most obvious mutations, we found genetic alterations in over 50% of the patients for *NOTCH1*, one of the best described events in T-ALL. All other reported recurrent mutations (among others *PTEN*, *PHF6*, *BCL11B*, or *WT1*) occurred in less than 20% of adult T-ALL patients[33]. The frequency of *NOTCH1* mutations as well as mutation rates for other well established genes like *WT1*, *FBXW7*, or *BCL11B* were in the range of previously reported incidences[33]. Another frequent alteration, genomic deletion of *CDKN2A*, was, however, not covered by our approach.

We also confirmed recurrent mutations in *DNM2*, *PHF6*, *PTEN*, *JAK3*, and *RUNX1*, which were only very recently discovered. The cadherins *FAT1* and *FAT3*, mutated in ETP-ALL[22], have not yet been described in non-ETP T-ALL of adults and were identified by our approach to be recurrently mutated across all subgroups of adult T-ALL. *FAT1* and its mutational inactivation have been linked to activation of the WNT pathway in solid tumors and to chemoresistance in chronic lymphocytic leukemia[48,49] and could serve as an attractive therapeutic target.

Furthermore, we found a high rate of mutations in *MLL2*, a histone methyltransferase, frequently mutated in various types of B-cell lymphomas[41-43]. Like in B-cell lymphomas, *MLL2* mutations were distributed over the entire gene without any obvious hot-spot region[41,50]. Interestingly, another histone methyltransferase, *WHSC1 (*also known as *MMSET/NSD2)*, was recurrently mutated in T-ALL and, although in a small number of patients, mutually exclusive within *MLL2*. *WHSC1*, associated with the so called Wolf-Hirschhorn syndrome[51], was only very recently found to be mutated in pediatric ALL, particularly in t(12;21) ETV6-RUNX1 ALL[45,46], as well as in mantle cell lymphoma[42]. These results together with mutations in the PRC2 complex and in genes involved in DNA methylation unravel a yet unreported high frequency (of over 25%) of alterations in epigenetic regulators in adult T-ALL. This is in line with other hematologic malignancies like acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) or diffuse large cell lymphoma[41,52,53]. These findings suggest that a very tight regulation of chromatin remodelling, especially for methylation of lysine 27 on histone H3, is required in physiological cell development and correct hematopoietic differentiation.

Interestingly, patients with an immature T-ALL immunophenotype showed a particular high frequency for mutations in epigenetic regulators and thus emphasize the similarity with myeloid malignancies. This is especially

striking in the subgroup of ETP-ALL as already described by Zhang and colleagues[21]. We were unable to confirm the high mutation rate in the PRC2 members described for pediatric patients, but we frequently found mutations in regulators of DNA methylation, possibly related to preexisting lesions in hematopoietic progenitors in the elderly[22,54]. Taken together, the high frequency of mutations in epigenetic regulators offers new insights and potential therapeutic applications e.g. of EZH2 inhibitors, histone deacetylase (HDAC) inhibitors or demethylating agents, which should be explored in clinical studies.

Another promising pathway for targeted therapies is the JAK/STAT pathway with frequent *JAK3* mutations (13%). This rate is higher than the reported frequency in pediatric ALL patients[21]. For *JAK1*, varying mutation rates (4-18%) have been published[24,55]; in our cohort we found 4% of *JAK1* mutations. In total, 18% of all our adult T-ALL patients carried alterations in the JAK/STAT pathway, predominantly in high-risk patients with an immature immunophenotype that might benefit from the application of molecular directed therapies, including JAK inhibitors[56].

Interestingly, mutations in the spliceosome, which are frequently found in MDS and in subgroups of AML[52,53], were virtually absent in T-ALL patients throughout all subgroups. Therefore, other elementary cellular processes might play a role in T-ALL. In a recent study, alterations in posttranslational mechanisms were suggested[25]. Unfortunately, these findings were reported after the design of our study and genes of interest, like *RPL5*, *RPL10*, or *CNOT3*, were not included in our gene panel.

In contrast to the work of de Keersmaecker and colleagues, we did not observe an age dependent distribution of mutation frequencies[25]. However this is likely due to our study design focused on candidate genes potentially enriched for driver mutations. The difference in the mutation frequencies in unbiased whole exome approaches and the frequencies in selected gene panels raises the question, whether the higher rate of mutations in prima vista not T-ALL associated genes simply reflects the altered hematopoiesis in elderly or possesses itself a leukemogenic potential[57-59].

NGS techniques are becoming widely available and are about to guide treatment decisions. This offers the opportunity not only to identify targets but also to unravel the spectrum of subclonal architecture that likely affects the response to targeted therapies. In addition, the mutational spectrum of leukemic cell changes during the progression of the disease and relapses are frequently harbored in preexisting subclones[47,57]. It has been shown that specific mutations, which are only present in a minor subclone at diagnosis, could lead to relapse due to chemotherapy resistance[60,61]. Therefore, the sole assessment of mutated genes might insufficient to select an optimal targeted therapy and determination of mutation frequency might be necessary to predict responses and the risk of relapse. This additional level of complexity in describing mutational landscapes for each individual patient is explored in our study and emphasizes that reported drivers not only occurred in the founding clone, but also in subclones. Thus, the sole restriction to gene panel assays for diagnostic purposes will likely not be sufficient to capture the wide clonal diversity and thus will likely miss mutations in the founding clone and even more in the subclones[47].

Adult T-ALL reveals a highly heterogeneous and individual spectrum of candidate gene mutations. Here, we provide an original and comprehensive overview of recurring mutations that unravel altered pathways enriched in specific leukemic subgroups. In addition, we identified novel candidate genes with potential therapeutic implications (*FAT1*, *MLL2*, *HERC1*). These mutations have to be further validated in larger patient cohorts accompanied by functional assays regarding their value as potential therapeutic targets. The identification of individual lesions in daily clinical routine, their clonal evolution, and the incorporation of highly individualized therapies in study trials will be a future challenge.

PATIENTS AND METHODS

Patients and treatment

We investigated bone marrow samples from 81 adult T-ALL patients with material sent to the reference laboratory of the GMALL study group and with sufficient genomic DNA quality, quantity and blast count (>80%) to perform NGS (Supplementary Table S4). Immunophenotyping of fresh samples was centrally performed in the GMALL reference laboratory at the Charité, University Hospital Berlin, Germany. Immunophenotyping was carried out as previously described[62,63]. T-lineage leukemia was subclassified into pre-T-ALL or early T-ALL (cyCD3+, CD7+, CD5+/−, CD2−, sCD3−, CD4−/+, CD8−/+, CD1a− or cyCD3+, CD7+, CD5−, CD2+, sCD3−, CD4−, CD8−, CD1a−), thymic T-ALL (cyCD3+, CD7+, CD5+/−, CD2+/−, sCD3+/−, CD4+, CD8+, CD1a+), and mature T-ALL (cyCD3+, CD7+, CD5+, CD2+, sCD3+/−, CD4+/−, CD8+/−, CD1a−). The immature subtypes of pre-T-ALL and early T-ALL are merged to a combined early T-ALL group. In addition, patients were classified to have an ETP-ALL within early T-ALL according to the criteria originally used by Couston-Smith and colleagues[36]. In detail, all samples were positive for cyCD3 and CD7, with absence of CD1a and CD8 (less than 5% of all lymphoblasts were positive), and weak expression of CD5 (i.e. less than 75% of all lymphoblasts were positive). Furthermore, the immunophenotype was characterized by

Table 2: Characteristics of the investigated adult T-ALL patients. Abbreviations: WBC, white blood cell count; TCR, T cell receptor

Number of patients		81
Sex	male	67
	female	14
Age(years)	Median	35
	Range	17-73
WBC (/nl)	Median	41.9
	Range	0.8-332
Mediastinal mass (n=62)	yes	42
	no	20
TCR rearrangement (n=75)	yes	53
	no	22
Immunophenotype	thymic	40
	mature	15
	early	26

the expression (i.e. more than 25% of the lymphoblasts positive) of at least one myeloid or stem cell marker (CD13, CD33, CD65, CD117, CD34, HLA-DR).

Patients' characteristics

Of the 81 adult T-ALL patients examined in this study, 40 patients showed an immunophenotype of thymic T-ALL, 15 of mature T-ALL and 26 of early T-ALL, and amongst the latter group, 20 had an ETP-ALL immunophenotype. The median age was 35 years (range 17-73) and 83% of the patients were male. The median white blood cell count (WBC) at diagnosis was 41.9/nL (range 0.8-332; Table 2). For all 81 samples the TCR rearrangement status was available[64].

Selection of candidate genes

The 88 genes selected for targeting sequencing are listed in the supplement (Supplementary Table S2). We selected genes known to be recurrently mutated in T-ALL, but also other genes, frequently mutated in BCP-ALL, (AML), and (MDS) were incorporated into the analysis[21,25,52,65]. Furthermore, genes with functions in epigenetic regulations, like members of the (PRC2) or in the splicing machinery, were included. Also, we selected candidate genes, based on our analysis of five exomes of adult ETP-ALL[22]. The targeted region of the 88 genes covered 1427 coding exons and 311 Kb of sequence (Supplementary Table S2).

Targeted sequencing of candidate genes

We constructed libraries from 3 µg of genomic DNA, which were labeled by barcode indices (length: 6 bp). Customized biotinylated RNA oligo pools (SureSelect, Agilent) were used to hybridize the target regions comprising the 88 selected genes. We performed 76-bp paired-end sequencing on an Illumina Genome Analyzer IIx platform. Reads were mapped to NCBI hg19 RefSeq. For a variant call, we required at least a read depth of 30, an allele frequency of 20% and an average base calling quality of Q13. Polymorphisms annotated in dbSNP 135 were excluded (Supplementary Figure S2).

Statistical analyses

Differences in the clinical characteristics were tested by the Pearson χ^2 test resp. Fisher test. Differences in the mutation rate were analyzed by the Pearson χ^2 test. For all tests a P-value < 0.05 (two-sided) was considered to indicate a significant difference. All calculations were performed using the SPSS software version 17 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism® software version 5 (GraphPad Software Inc., La Jolla, CA, USA).

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7. DISKUSSION

Die T-ALL des Erwachsenen stellt nach wie vor eine klinische Herausforderung bezüglich einer Therapieoptimierung dar. Fünf-Jahres-Überlebensraten von ca. 50% auch für jüngere Erwachsene sind immer noch unbefriedigende Ergebnisse. Wie in der Einleitung dargestellt, sind für die T-ALL im Gegensatz zur B-Vorläufer-ALL noch keine zielgerichteten Therapien etabliert. Hoffnung auf eine Besserung entsteht durch den zunehmenden, rasanten molekularen Erkenntnisgewinn mittels Hochdurchsatzverfahren mit der Aussicht auf identifizierbare Zielstrukturen. Hierbei sind auf Grund der zu erkennenden molekularen Heterogenität, vergleichbar zu anderen Tumorentitäten, noch gemeinsame Muster zu identifizieren und für die Klinik gangbare Therapiealgorhythmen zu entwickeln. Dies sei im Folgenden am Beispiel der in dieser Arbeit beschriebenen Untersuchungen diskutiert.

Die Gruppe der ETP-ALL ist zuerst in pädiatrischen T-ALL-Patienten mit einer Frequenz von ca. 10% beschrieben worden (Coustan-Smith *et al*, 2009). Wir konnten diese basierend auf dem Immunphänotyp ($CD7^{+}$, $CD5^{weak}$, $CD1^{-}$, $CD8^{-}$, Koexpression von Stammzell- (CD34, CD117, HLA-DR) oder myeloischen (CD33, CD13, CD14) Markern mit einer vergleichbaren, leicht niedrigeren Frequenz von ca. 8% in der erwachsenen T-ALL nachvollziehen. Initial wurde in der pädiatrischen T-ALL für diese Patientenkohorte eine so geringe Überlebensrate beschrieben, dass eine allogene Stammzelltransplantation in erster kompletter Remission angeraten wurde. Auch wenn diese Schlussfolgerung mittlerweile Gegenstand einer umfassenden Diskussion ist (Jain *et al*, 2016), lässt sich am Beispiel der ETP-ALL ein prinzipieller Unterschied zwischen pädiatrischer und erwachsener T-ALL demonstrieren. In der erwachsenen T.ALL ist die ETP-ALL eine Untergruppe der frühen T-ALL, für die bereits als Hochrisikogruppe eine allogene Stammzelltransplantation in erster kompletter Remission empfohlen ist. Somit erfolgt trotz der vergleichbar schlechten Prognose initial keine veränderte Therapieempfehlung. Allerdings ermöglicht die Identifizierung einer ETP-ALL auf Grund ihrer molekularen Charakteristika im Rezidiv nach allogener Stammzelltransplantation zusätzliche therapeutische Optionen. Diese basieren insbesondere auf der hohen Frequenz von FLT3-ITD-Mutationen mit bereits aus der AML bekannten zielgerichteten Therapien mittels Tyrosinkinaseinhibitoren. Zusätzlich ermöglichen die molekularen Eigenschaften, die eher einer AML entsprechen (Expression von Stammzellmarkern, Oberflächenexpression von myeloischen Markern, Mutationsspektrum), auch

Therapieoptionen, die sich von der Therapie einer AML ableiten, als gangbare Alternative in der Rezidivsituation ohne wirklich erfolgsversprechende Standardtherapien.

Ein zunächst eher abstrakter Gewinn besteht im Stammzellmodell einer ETP-ALL. Sie besitzt eine Anzahl von Eigenschaften, die auf eine sehr frühe, unreife Zelle als Ursprung der Transformation hindeutet. Somit unterscheidet sich diese Gruppe in vielerlei Hinsicht fundamental von der einer T-ALL, obwohl sie sich sowohl morphologisch als auch immunzytologisch dieser eindeutig zuordnen lässt. Dies verleiht ihr einen Modellcharakter, in der Therapieformen unabhängig von der eigentlichen Tumorentität auch an Hand von molekularen Veränderungen vorgenommen werden könnten. Erkenntnisse, die sich gegebenenfalls auch auf andere unreife Formen der akuten Leukämie mit einem oft schwer zu therapierenden aggressiven Verlauf, wie eine sehr frühe AML M0, eine pro B-ALL oder biphänotypische akute Leukämien, übertragen lassen.

Zusammengenommen haben die spezifischen, biologischen Merkmale einer ETP-ALL zur vorläufigen Aufnahme in die WHO-Klassifikation von lymphatischen Neoplasien geführt, auch wenn die prognostische Bedeutung dieser Subgruppe weiterhin diskutiert wird (Arber *et al*, 2016).

Wie die Erkenntnis von spezifischen molekularen Alterationen zu möglichen Therapieoptionen führen kann, war in der Einleitung bereits am Beispiel der Ph+-like ALL diskutiert worden. Aber auch für die ETP-ALL mit einer aberranten Aktivierung des JAK/STAT-Signalwegs wurde über Mausmodelle der Einsatz von Ruxolitinib, einem unspezifischen Inhibitor des JAK/STAT-Signalwegs, als effektive anti-leukämische Therapie gezeigt (Maude *et al*, 2015). Damit stehen zusammen mit einem *FLT3*-Inhibitor bereits zwei zielgerichtete Therapieoptionen für Subgruppen der T-ALL in einer Rezidivsituation zur Verfügung. Wie in den obigen Arbeiten gezeigt, ist der JAK/STAT-Signalweg nicht nur innerhalb der ETP-ALL, sondern auch in der gesamten T-ALL regelmäßig affektiert. Insbesondere *JAK3* ist in über 10% aller Fälle betroffen. Auch für diese Fälle stehen mit dem spezifischen JAK3-Inhibitor Tofacitinib, der erste dieser Klasse, Ruxolitinib oder sogar einer Kombination beider JAK-Inhibitoren, effektive Therapieoptionen zur Verfügung (Degryse *et al*, 2014).

NGS-Untersuchungen mittels WGS und WES haben die vorher nicht wahrgenommene Rolle von Modifikationen von epigenetischen Regulatoren in der akuten Leukämie enthüllt. Dies ist am prominentesten in der AML für *DNMT3A* und *TET2* als DNA-

Methylierungsmodifikatoren gezeigt wordem (Ley *et al*, 2009). Diese scheinen erste, frühe Modifikationen in der Leukämogenese zu sein, wie auch das Vorkommen in der Hämatopoese älterer, gesunder Personen zeigt (Genovese *et al*, 2014; Jaiswal *et al*, 2014). Wir konnten *DNMT3A*-Mutationen auch in der T-ALL zeigen, wobei diese eher bei älteren Patienten vorkommen und im Kindesalter zuvor nicht gefunden wurden (Neumann *et al*, 2012). Das legt die Vermutung nahe, dass sich die T-ALL auf der Basis einer präleukämischen *DNMT3A*-Läsion entwickelt hat. Neben Alterationen von DNA-Methyltransferasen zeigen sich aber auch Mutationen in Histonmodulatoren wie *MLL2*, *EZH2*, *SUZ12*, *JARID2* oder *EP300*. Insgesamt waren in über 35% aller erwachsenen T-ALL-Patienten epigenetische Regulatoren affektiert (Neumann *et al*, 2014). Inwieweit sich diese als therapeutische Zielstrukturen verwenden lassen, ist momentan noch unklar. Für die AML sind die hypomethylierenden Substanzen Decitabine oder 5-Azacytidine zugelassene Therapien. Für die T-ALL sind diese noch nicht evaluiert, obwohl interessanterweise T-ALL-Zelllinien eine besonders hohe Sensitivität gegenüber Decitabine zeigen (unveröffentlichte Daten, AG Baldus). Neben diesen besser erforschten *DNMT3A*-Inhibitoren sind aber zahlreiche weitere Therapieoptionen in frühen klinischen Testungen, wie Inhibierung der H3K70-Methyltransferase *DOT1L*, BRD4-Inhibitoren, HDAC-Inhibitoren oder auch AGI-6780 als spezifische Therapieoption für IDH-mutierte akute Leukämien (für eine Übersicht z.B. Peirs *et al*, 2014).

Bei all diesen besprochenen zielgerichteten Therapieoptionen ist der am häufigsten betroffene NOTCH-Signalweg mit aktivierenden *NOTCH1* und *FBXW7*-Mutationen in über 50% aller T-ALL-Patienten noch gar nicht berücksichtigt. In der Entwicklung von Gamma-Sekretase-Inhibitoren zeigen sich die Hoffnungen und Schwierigkeiten einer zielgerichteten Therapie. Erste Therapieversuche mit MK-0752 zeigten neben einem nur sehr mäßigen Therapieansprechen vor allem eine dosislimitierende, gastrointestinale Toxizität, die zu einer langen Verzögerung führten (Deangelo *et al*, 2006). Neuere Ansätze, die sich in frühen klinischen Studien befinden, scheinen diese initiale Limitierung überwinden zu können, wie sie z.B. für BMS-906024 mit einem Ansprechen von acht aus 25 Patienten in einer Phase I-Studie bei nur minimaler gastrointestinaler Toxizität berichtet wurde (Zweidler-McKay *et al*, 2014). Hierzu gesellen sich weitere Therapieansätze wie TKI in NUP212-ABL1-rearrangierten, BCL2-Inhibtion mit ABT-199 in *BCL2* überexprimierenden oder PI3K-mTOR-Inhibitoren in PTEN deletierten T-ALL-Fällen (Litzow *et al*, 2015).

Zusammen mit weiteren, am Beispiel von BCL11B und FAT1 in dieser Arbeit diskutierten Markern, die sich durch eine aberrante Expression auszeichnen, und damit ebenfalls als Angriffspunkt, aber auch als Verlaufsparameter zur Verfügung stehen, scheint es nicht utopisch, in einer Großzahl der T-ALL-Patienten therapeutische Zielstrukturen zu identifizieren. Diese potentiellen Strukturen sind durch den exponentiell verlaufenden Datenzugewinn durch moderne Hochdurchsatzverfahren mittlerweile zahlreich. Im Verhältnis hierzu sind die tatsächlich zum Einsatz gekommenen Therapeutika zahlenmäßig gering und oft auf anekdotische Fallberichte beschränkt. Die Gründe für dieses Ungleichgewicht sind zahlreich und stellen die eigentliche Herausforderung der Translation der Erkenntnisse über die molekularen Mechanismen der Leukämogenese in die klinische Behandlung dar.

Zunächst ist der Wissensstand vieler molekularen Alterationen noch sehr deskriptiv und für viele ist die funktionelle Rolle in der Leukämogenese noch ungeklärt. Allerdings scheint dies nicht der limitierende Faktor zu sein. Zum einen sind viele Mausmodelle im Entstehen, die eine bessere Einschätzung der Kausalität liefern werden. Zum zweiten ist die Frage, ob Technologien, die auch ein Medikamenten-Screening im großen Maßstab ermöglichen, nicht relativ zeitnah zur Verfügung stehen werden, die ein Ansprechen auf bestimmte Therapien prädiktieren können. Als Beispiel seien hierfür CRISPR/cas9-screens (Shi *et al*, 2015) und small molecule screens genannt. Hier muss allerdings, ebenso wie für die Etablierung neuer Therapien, eine ausreichende Evidenz geschaffen werden, um eine Integration in die alltägliche Praxis zu gewährleisten. Wenn man bedenkt, dass in der T-ALL seltene molekulare Alterationen deutschlandweit oft nur wenige Fälle pro Jahr einschließt, ist unmittelbar klar, dass in bestehenden Studienkonzepten eine Testung nur unzureichend verwirklicht werden kann. Hieraus resultiert die Notwendigkeit, dass neuere Studienmodelle wie „umbrella“ oder „basket“-Designs konsequent verwirklicht werden müssen. Somit müssen zum einen Krankheitsentitäten gegebenenfalls entsprechend ihres molekularen Hintergrunds unterschiedlich behandelt werden. Dies ist für einige Entitäten bereits der Fall (Mammakarzinom mit HER2-Amplifikationen und Östrogenrezeptorexpression oder Lungenkarzinom mit EGFR-Mutationen oder ALK-Fusionsgenen), für viele andere, insbesondere seltene Veränderungen, noch unzureichend evaluiert. Zum zweiten müssen gemeinsame molekulare Alterationen in verschiedenen Krankheitsentitäten auf gemeinsame Therapiemodalitäten evaluiert werden. Dies verlangt neben dem Bewusstsein über vorhandene Therapieoptionen in anderen Krankheitsentitäten auch die

Etablierung von molekularen Tumorboards in Ergänzung zu den entitätsspezifischen Tumorboards und die Möglichkeit, Patienten standortübergreifend in Therapiestudien einschließen zu können. Im Rahmen der GMALL-Studiengruppe mit der nahezu populationsbasierten Zentrenrekrutierung stehen für die ALL modellhaft Rahmenbedingungen zur Verfügung, in welchem auch die diagnostischen Herausforderungen hinsichtlich einer gezielten, aber dennoch umfassenden molekularen Charakterisierung verwirklicht werden können und teilweise schon verwirklicht worden sind.

Auch wenn eine grundlegend veränderte, zielgerichtete, chemofreie Therapie der T-ALL zur Zeit sicherlich illusorisch ist und angesichts der Nebenwirkungen mancher „zielgerichteten“ Tyrosinkinaseinhibitoren vielleicht auch gar nicht gewünscht ist, eröffnet der Zugewinn an molekularer Erkenntnis, wie er in dieser Arbeit ausschnittsweise besprochen wurde, doch neue Therapieoptionen zunächst in der rezidivierten Situation ohne anderweitige Standardtherapien. Verbunden mit der Hoffnung auf eine in Zukunft, in Studien zu verifizierende, höhere Rate an kurativ zu behandelnden T-ALL-Patienten.

8. ZUSAMMENFASSUNG

Die molekulare Charakterisierung der T-ALL ermöglicht neben einem besseren Verständnis der Leukämogenese auch verschiedene Formen der Therapieoptimierung. Diese sind in der vorliegenden Arbeit zunächst für eine subgruppenorientierte Risikostratifizierung mit der Identifizierung charakteristischer molekularer Eigenschaften am Beispiel der ETP-ALL gezeigt worden. Hierbei konnten folgende Resultate beschrieben werden:

TEIL I:

- Die Frequenz der ETP-ALL, definiert durch den Immunphänotyp, liegt bei ca. 8% aller erwachsenen T-ALL-Patienten.
- Die Prognose der ETP-ALL im Erwachsenenalter ist vergleichbar mit der Prognose der Hochrisikogruppe der frühen T-ALL.
- Die ETP-ALL weist sowohl Charakteristika einer Stammzellleukämie als auch einer Leukämie mit myeloischer Differenzierung auf.
- Es findet sich eine hohe Rate an *FLT3*-ITD-Mutationen, die innerhalb der T-ALL nahezu ausschließlich in der Subgruppe der ETP-ALL auftreten.
- Die myeloischen Eigenschaften sowie die hohe Rate an *FLT3*-Mutationen eröffnen neue therapeutische Optionen.

Eine weitere Strategie ist die Identifizierung von Patienten, die trotz eines Standardrisikos einen ungünstigen Verlauf aufweisen und nach erfolgter Behandlung rezidivieren. Dies wurde am Beispiel des T-Zell-Transkriptionsfaktors *BCL11B* sowie des Proto-Cadherins *FAT1* untersucht:

TEIL II:

- Die niedrige Expression von *BCL11B* identifiziert Patienten mit einer ungünstigen Prognose innerhalb der thymischen T-ALL.
- *BCL11B* ist in 14% aller erwachsenen T-ALL-Patienten mutiert, wobei diese nahezu ausschließlich die funktionellen Zinkfingerdomänen betreffen.
- Das Expressionsprofil von *BCL11B* in der T-ALL reflektiert die physiologische T-Zell-Entwicklung.
- *FAT1* ist sowohl in der T-ALL als auch der B-Vorläufer-ALL aberrant, reifungsabhängig exprimiert.
- *FAT1* ist in ca. 12 % aller T-ALL-Patienten mutiert.

Neben der optimalen Risikostratifizierung ist die Identifizierung von Zielstrukturen, die potentiell therapeutische Optionen ermöglichen, von zentraler Bedeutung. Dies wurde unter dem Einsatz von NGS-Technologien im dritten Teil der Arbeit am Beispiel der ETP-ALL mittels WES sowie in einer größeren Kohorte von 81 T-ALL-Patienten mittels Genpanel verfolgt:

TEIL III:

- Es konnten erstmals somatische Mutationen für *DNMT3A* in der T-ALL beschrieben, und in einer größeren Kohorte für 16% der Patienten bestätigt werden.
- Das Mutationsspektrum von erwachsenen ETP-ALL-Patienten unterscheidet sich von dem der pädiatrischen ETP-ALL-Patienten.
- In der Untersuchung von 88 Leukämie-assoziierten Genen konnten in über 90% der T-ALL-Patienten Alterationen nachgewiesen werden.
- In einer Vielzahl der Fälle waren Signalwege betroffen, die potentiell therapeutische Optionen ermöglichen.
- Die Beschreibung des Mutationsspektrums bildet eine Basis für die Implementierung zielgerichteten Therapien.

Insgesamt wurden in dieser Arbeit molekulare Strukturen sowohl in Hinsicht auf eine Optimierung der Risikostratifizierung als auch im Hinblick auf zielgerichtete Therapien in der T-ALL des Erwachsenen untersucht. Hierbei konnten geeignete Kandidaten identifiziert werden. Die Herausforderung besteht in der Translation dieses Wissens in praktische, klinische Therapiealgorhythmen.

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10. ABKÜRZUNGEN

ALL	Akute lymphoblastische Leukämie
BFM	Berlin, Frankfurt, Münster
AML	Akute myeloische Leukämie
BCP-ALL	B-Vorläufer ALL
CML	Chronisch myeloische Leukämie
EGIL	European Group for the Immunological Characterization of Leukemias
ETP-ALL	„early T-cell precursor“-ALL
GMALL	German multicenter ALL study group
ITD	Internal tandem duplication
MRD	Minimale Resterkrankung
PH+-ALL	Philadelphiachromosom-positive ALL
PRC	Polycomb repressor complex 2
RNAseq	RNA sequencing
T-ALL	T-Zell akute lymphoblastische Leukämie
T-LBL	T-lymphoblastisches Lymphom
WES	“whole exome sequencing”
WGS	“whole genome sequencing”

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12. ERKLÄRUNG

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

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