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Klinische Relevanz phänotypischer und blutbasierter Biomarker beim Mammakarzinom

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1. In die Habilitationsschrift eingebundene eigene Arbeiten

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2. Einleitung

Die Diagnostik und Therapie des Mammakarzinoms durchlebten in den letzten Dekaden einen großen Wandel. Einerseits spielen die biologischen Eigenschaften der Erkrankung bei der Prognoseeinschätzung und Planung der zielgerichteten Therapie eine immer wichtigere Rolle, während die klassischen TNM-Stadium basierten Prognosefaktoren in den Hintergrund geraten [1, 2]. Anderseits wird das Mammakarzinom nicht mehr als lokales Geschehen, sondern bereits in frühen Stadien als eine systemische Erkrankung betrachtet [3, 4]. Die Suche nach weiteren phäno- und genotypischen blut- und primärtumorbasierten Markern, die eine bessere Einschätzung der Prognose und/oder eine Prädiktion des Therapieansprechens erlauben, stellt vor diesem Hintergrund einen zentralen Schwerpunkt der gegenwärtigen Mammakarzinomforschung dar.

Phänotypbasierte Biomarker beim Mammakarzinom

Der Hormonrezeptortatus und der HER2-Status stellen die klassischen sowohl prognostischen als auch prädiktiven Faktoren beim Mammakarzinom dar und die endokrine bzw. die anti-HER zielgerichtete Therapie gehören zu den Therapiestandards in der primären und metastasierten Situation [5]. Neue prädiktive Faktoren für spezielle Risikogruppen, wie der PD-L1 Status für die Immuntherapie beim tripel-negativen Mammakarzinom [6] oder der BRCA-1/2-Status für die Therapie mit PARP-Inhibitoren bei Patientinnen mit HER2-negativem Mammakarzinom [7, 8], haben die Diagnostik und Therapie der Erkrankung in den letzten Jahren ergänzt und die Prognose dieser Patientinnen signifikant verbessert [5].

Einen aktuell diskutierten Biomarker stellt in diesem Kontext der Androgenrezeptor(AR)-Status dar. Der AR wird bei über 70% aller und bis zu 43% der tripel-negativen Mammakarzinome exprimiert [9-13]. Trotz vieler Studien bleibt seine Rolle beim Mammakarzinom bis heute nicht abschließend geklärt [14]. In-vitro-Untersuchungen zufolge ist der proliferative Effekt von Androgenen beim Mammakarzinom vom Tumorsubtyp abhängig [15], [16]. Während die proliferative Wirkung beim HER2-positiven und tripel-negativen Mammakarzinom klar erscheint [17], kann der AR bei ERα-Positivität je nach Stärke der ERα Co-Expression und der Anwesenheit des entsprechenden Liganden proliferativ oder anti-proliferativ wirken [18], [19], [20]. Interessanterweise konnte eine Assoziation zwischen AR-Überexpression und Tamoxifen-Resistenz auf Hormonrezeptor-positiven Mammakarzinomzellen demonstriert werden, welche durch eine anti-androgene Behandlung reversibel war [21]. Gleichzeitig zeigen die AR-positiven Mammakarzinome in klinischen Studien ein schlechtes Ansprechen auf eine zytotoxische Therapie [11, 22]. Vor diesem Hintergrund stellt der AR ein potentielles

Behandlung-Target dar, weshalb die Effektivität einer zielgerichteten antiandrogenen Therapie bei AR-positiven Mammakarzinompatientinnen derzeit in zahlreichen Studien untersucht wird [14]. Die ersten Ergebnisse bei metastasiertem tripel-negativen Mammakarzinom mit AR-Überexpression sind mit klinischen Ansprechraten von bis zu 25% vielversprechend [23], [24]. Der AR-Status beim Mammakarzinom wird nicht routinemäßig bestimmt, sodass im Falle dieser Fragestellung in der metastasierten Situation die Archivproben des Primarius reevaluiert werden oder eine invasive Biopsie einer der Fernmetastasen erfolgen muss.

Einen interessanten und bis dato wenig erforschten Biomarker beim Mammakarzinom stellt die neuroendokrine Differenzierung dar. Neuroendokrine Mammakarzinome (NEMCA) machen unter 1% der neuroendokrinen Neoplasien (NEN) aller Organsysteme aus [25, 26] und stellen gleichzeitig eine extrem seltene Form des Mammakarzinoms dar. Aufgrund der häufigen Änderungen in der Definition dieser Läsionen in den letzten 2 Dekaden variieren in der Literatur die Angaben zur Häufigkeit der NEMCA unter allen invasiven Mammakarzinomen zwischen 0.1 und 20% [27]. Die Bestimmung der neuroendokrinen immunhistochemischen Marker (Synaptophysin und Chromogranin A) erfolgt nicht routinemäßig, sodass die korrekte Diagnosestellung und Erörterung der klinischen Charakteristika dieser Tumore herausfordernd sein können. Die bisherigen retrospektiven Analysen konnten die neuroendokrine Differenzierung beim Mammakarzinom als ungünstigen Prognosefaktor identifizieren [28]. Spezielle therapeutischen Ansätze sind bei NEMCA bis dato nicht etabliert worden. Eine Ausnahme stellt das kleinzellige neuroendokrine Mammakarzinom (*small cell neuroendocrine carcinoma,* SCNEC) dar, eine hoch aggressive NEMCA-Form, die in Analogie zum kleinzelligen Bronchialkarzinom behandeln werden kann [29], [30]. Gleichzeitig können NEMCA spezifische Somatostatinrezeptoren (SSTR) exprimieren, wobei der SSTR2A eine besondere Relevanz zu haben scheint. Eine SSTR2A-Positivität erlaubt einerseits eine Somatostatin-Analoga basierte Diagnostik (z.B. 68Gallium-DOTATOC-PET-CT oder 111Indium-Octreotid-SPECT), andererseits wird bei SSTRA2A-positiven NEN anderer Organe, bespielweise des gastrointestinalen Systems, eine gezielte Therapie mit Somatostatin-Analoga angewandt [31]. Die Häufigkeit der SSTR2A-Expression sowie der SSTR-basierten Diagnostik bei NEMCA wurden bislang kaum beschrieben [32]. In einer durchgeführten Phase III Studie konnte kein Benefit durch die zusätzliche Therapie mit Somatostatin-Analoga beim Mammakarzinom gezeigt werden [33]. Allerdings wurde der SSTR2A-Status der Tumore in dieser Analyse nicht berücksichtigt.

Das Phänomen der Tumorzelldissemination

Die Hypothese zur hämatogenen Streuung von Tumorzellen beim Mammakarzinom wurde bereits Ende des neunzehnten Jahrhunderts von Paget gestellt [34]. Trotzdem wurde das Mammakarzinom lange Zeit nach der sog. „Halsted-Doktrin“ als eine lokale Erkrankung betrachtet, mit einer möglichst radikalen Operation als einzige Heilungsmöglichkeit. Dieses Paradigma wurde von der sog. „Fischer-Doktrin“ abgelöst, welche die Brustkrebserkrankung schon in frühen Stadien als ein systemisches Geschehen ansieht [35]. Mittlerweile ist bewiesen, dass die Tumorzelldissemination einen wichtigen Schritt im Prozess der Metastasierung darstellt. So können isolierte Tumorzellen im Blut oder Knochenmark von nicht-metastasierten Mammakarzinompatientinnen nachgewiesen werden und gelten als Surrogatmarker der minimalen Tumorresterkrankung (MRD; minimal residual disease) und potenzielles Ziel der zukünftigen Therapieansätze [36, 37]. Interessanterweise können die isolierten Tumorzellen auch bei Patientinnen mit präinvasiven Läsionen der Mamma detektiert werden [38], [3]. Während die Rolle dieser Zellen beim in situ Mammakarzinom bis dato unklar bleibt, ist die unabhängige, prognostische Relevanz der Tumorzelldissemination beim nicht-metastasierten invasiven Mammakarzinom sowohl für die disseminierten Tumorzellen (DTC; disseminated tumor cells) im Knochenmark [39] als auch für die zirkulierenden Tumorzellen (CTC; circulating tumor cells) im peripheren Blut mit dem höchsten Level der Evidenz bestätigt [40]. Gleichzeitig entwickeln nicht alle DTC- oder CTC-positive Patientinnen ein Krankheitsrezidiv [41]. Nach der Theorie der metastatischen Ineffizienz („*metastatic inefficiency*“) werden die meisten der in die Blutbahn gelangenden Tumorzellen vom Immunsystem oder durch mechanische Scherkräfte des Blutes eliminiert [42], [43]. Nur eine kleine Subpopulation kann in der Blutbahn oder in den sogenannten sekundären „homing sites“, z.B. Knochenmark, persistieren und die spätere Fernmetastasierung verursachen. Welche Eigenschaften dieser Zellen ihr metastatisches Potential bestimmen wurde *bis dato* nicht endgültig geklärt. Somit stellt nicht nur der Nachweis dieser Zellen, sondern auch deren weitergehende Charakterisierung den zentralen Punkt der aktuellen Mammakarzinomforschung dar.

Bedeutung der Tumorzelldissemination beim metastasierten Mammakarzinom

Auch beim metastasierten Mammakarzinom (MBC) konnte der Nachweis zirkulierender Tumorzellen im peripheren Blut als unabhängiger negativer Prognosefaktor in zahlreichen Studien gezeigt werden [44]. Eine gepoolte, retrospektive Analyse von 2436 MBC-Patientinnen aus dem Jahr 2019 konnte die unabhängige prognostische Bedeutung dieser Zellen erneut bestätigen. Ein cut-off Wert von mehr als 5 CTCs in 7,5 ml peripheren Blut wurde

in den meisten eingeschlossenen Analysen angegeben und gilt als prognostisch signifikant (medianes OS: 36,3 bei < 5 CTCs vs. 16,0 Monate bei ≥ 5 CTCs, $p < 0,0001$) [45].

Über die prognostische Bedeutung hinaus kann anhand der CTC-Dynamik das Therapieansprechen bei MBC beurteilt werden [46]. Ein CTC-Abfall unter 5 CTCs / 7,5ml Blut bereits nach dem ersten Zyklus der Therapie wurde in mehreren Studien beobachtet und war mit einem besserem progressionsfreien und Gesamtüberleben assoziiert [47-49]. Darüber hinaus kann einer prospektiven multizentrischen Untersuchung zufolge das Therapieansprechen anhand der CTC-Dynamik besser als mit konventioneller radiologischer Bildgebung vorausgesagt werden. Persistierende hohe CTC-Zahlen waren in dieser Analyse trotz des Ansprechens in der Bildgebung mit einem schlechten klinischen Outcome verbunden [50]. Bislang ungeklärt bleibt weiterhin die Frage nach der therapeutischen Konsequenz der Persistenz hoher CTC-Zahlen im metastasierten Setting. In der randomisierten Phase III SWOG-Studie wurde bei fehlendem CTC-Abfall nach dem ersten Zyklus der palliativen Chemotherapie auf ein anderes Regime geswitcht. Allerdings führte die CTC-Zahl-basierte Therapieumstellung nicht zur erhofften Verbesserung der Prognose. Es wird vermutet, dass CTC-Persistenz während der Chemotherapie eine Resistenz gegen konventionelle zytotoxische Behandlung reflektieren kann [49].

CTC-basierte Liquid Biopsy (LB)

Die prädiktiven Eigenschaften, die als Grundlage für die Therapiewahl beim Mammakarzinom dienen, können sich im zeitlichen Verlauf sowie unter Einfluss zahlreicher Therapien ändern [51]. Somit entsteht bei vielen Patientinnen eine therapierelevante Diskrepanz zwischen dem Phäno- und/oder Genotyp des Primarius und der Metastase. In einer großen Metaanalyse von 39 Studien wurde der Verlust des HR-Status oder HER2-Status bei 22,5% bzw. 21,3% der Patientinnen beschrieben, während in 9,5% der Fälle HER2-positive Metastasen trotz des HER2-negativen Primarius beobachtet wurden [52]. Aus diesem Grund wird im Falle der Fernmetastasierung eine Neubestimmung dieser Marker empfohlen. Den derzeitigen Goldstandard hierfür stellt eine Biopsie der Metastase dar [1]. Allerdings weist diese viele Limitationen auf, wie (1) fragliche Repräsentativität aufgrund der Untersuchung von nur kleinem Tumorareal, insbesondere bei polytoper Metastasierung, (2) Invasivität mit hoher Patientenbelastung und potenziellen Komplikationen oder (3) gelegentlich gar nicht vorhandener Biopsiemöglichkeit bei schlechter Zugänglichkeit der Metastase. In diesem Kontext kann eine nicht invasive Untersuchung der CTCs im peripheren Blut als sog. Liquid Biopsy dienen, unter der Annahme, dass CTCs nicht nur die aktuelle Last der Tumorerkrankung widerspiegeln, sondern auch den Phäno- und Genotyp der dominierenden

Tumorpopulation im Körper reflektieren. Die Implementierung einer CTC-basierten LB in die Therapieentscheidungen beim MBC wird derzeit intensiv erforscht.
In der randomisierten Phase III STIC CTC Studie wurde die Therapieentscheidung (endokrine Therapie vs. Chemotherapie) entweder nach üblichen klinischen Kriterien (*treatment of physician's choice*") oder nach CTC-Zahl (< 5CTCs vs. ≥ 5 CTCs) getroffen. Die auf der CTC-Zahl basierte Entscheidung war in der Analyse der ärztlichen Entscheidung nicht unterlegen: sowohl das progressionsfreie als auch das Gesamtüberleben waren in beiden Armen gleich [53]. In der randomisierten, multizentrischen Phase III DETECT III Studie wurde die klinische Relevanz einer auf dem CTC-Phänotyp basierten Therapieentscheidung bei MBC untersucht. Die Ergebnisse dieser Analyse wurden auf dem San Antonio Brustkrebs Symposium 2020 vorgestellt. Eine CTC-basierte, zielgerichtete Anti-HER-Behandlung mit Lapatinib war mit einem signifikanten Überlebensvorteil verbunden [54]. Trotz dieser vielversprechenden Ergebnisse bleibt die prädiktive Rolle des CTC-Phänotyps nicht endgültig geklärt. Eine weitere Charakterisierung dieser Zellen auf phäno- und genotypischer Ebene zur Identifizierung der Therapieresistenzen sowie Target-Strukturen für zielgerichtete Behandlung ist vor diesem Hintergrund essenziell.

Im Rahmen der vorliegenden Arbeit wurden phänotypische Biomarker beim Mammakarzinom basierend auf der Analyse des Primärtumors und/oder der disseminierten und zirkulierenden Tumorzellen in verschiedenen klinischen Situationen untersucht.

3. Ergebnisse: Darstellung der ausgewählten Originalarbeiten

3.1 HER2-Staus der persistierenden disseminierten Tumorzellen nach Abschluss der adjuvanten Therapie

Krawczyk N, Banys M, Neubauer H, Solomayer EF, Gall C, Hahn M, Becker S, Bachmann R, Wallwiener D, Fehm T. HER2 status on persistent disseminated tumor cells after adjuvant therapy may differ from initial HER2 status on primary tumor. Anticancer Res. 2009 Oct;29(10):4019-24. PMID: 19846945.

Einleitung:

Disseminierte Tumorzellen (DTC) können im Knochenmark von 10-15% der Patientinnen mit primärem Mammakarzinom nach Abschluss der adjuvanten Behandlung nachgewiesen werden. Diese persistierenden Tumorzellen sind mit einer schlechten Prognose assoziiert und werden als Surrogatmarker der minimal residualen Erkrankung angesehen [55]. Der Nachweis und die Charakterisierung dieser Zellen können möglicherweise Patientinnen identifizieren, die von den sekundären zielgerichteten Therapieansätzen profitieren.

Der humane epidermale Wachstumsfaktorrezeptor 2 (HER2) stellt einen der wichtigsten prognostischen und prädiktiven Faktoren in der Mammakarzinombehandlung dar [56]. HER2-positive Mammakarzinome weisen eine aggressive Tumorbiologie auf, können aber mit zahlreichen anti-HER Medikamenten zielgerichtet therapiert werden [5]. Eine Diskrepanz des HER2-Status zwischen Primärtumor und disseminierten Tumorzellen zum Zeitpunkt der Erstdiagnose wurde bereits beschrieben [57, 58]. Das Ziel der vorliegenden Arbeit war es (1) die Rate an Patientinnen mit persistierenden DTC nach Abschluss der adjuvanten Behandlung zu ermitteln und (2) den HER-2 Status dieser Zellen und des Primärtumors miteinander zu vergleichen, um herauszufinden, ob zielgerichtete anti-HER Therapie eine sinnvolle Option bei Patientinnen mit Tumorzellpersistenz darstellen könnte.

Methoden:

Bei 85 primären Mammakarzinompatientinnen wurde eine Knochenmarkspunktion intraoperativ sowie nach Abschluss der adjuvanten systemischen Behandlung (mediane Beobachtungszeit 13 Monate; 6-30 Monate) durchgeführt. Eine Immunfluoreszenz-Doppelfärbung wurde zur Identifikation von Zytokeratin-positiven Zellen sowie zur Bestimmung

ihres HER2-Status angewandt, basierend auf dem Konsens zum Tumorzellnachweis im Knochenmark von Patientinnen mit primären Mammakarzinom [37].

Ergebnisse:

31 von 85 Patientinnen (36%) wiesen einen positiven DTC-Status zum Zeitpunkt der Erstdiagnose auf, 14 von 85 Patientinnen (16%) waren DTC-positiv nach Abschluss der adjuvanten Behandlung. Bei 5 der 14 Patientinnen (36%) konnten HER2-positive DTC im Knochenmark nachgewiesen werden, obwohl nur 1 der 5 Primärtumore einen HER2-positiven Status hatte. Bei den restlichen 9 Patientinnen waren sowohl die persistierenden DTC als auch der Primärtumor HER2-negativ.

Schlussfolgerung:

Auch nach Abschluss der adjuvanten Therapie können HER2-positive DTC im Knochenmark von Patientinnen mit HER2-negativem Mammakarzinom nachgewiesen werden. Diese Patientinnen können möglicherweise von den sekundären zielgerichteten Anti-HER Ansätzen in der Adjuvanz profitieren.

3.2 Einfluss der Operation des Primärtumors auf die Detektionsrate und den Phänotyp von zirkulierenden Tumorzellen im peripheren Blut von primären Mammakarzinompatientinnen

Banys M*, **Krawczyk N*,** Becker S, Jakubowska J, Staebler A, Wallwiener D, Fehm T, Rothmund R. The influence of removal of primary tumor on incidence and phenotype of circulating tumor cells in primary breast cancer. Breast Cancer Research and Treatment 2012; 132(1):121-9
* both authors equally contributed

Einleitung:

Der prognostische Stellenwert der CTC im peripheren Blut von Patientinnen mit frühem, nicht metastasiertem Mammakarzinom konnte in zahlreichen Studien bestätigt werden [40]. Gleichzeitig wird die mittlere Halbwertzeit der in die Blutbahn gestreuten Zellen des Primärtumors auf wenige Stunden geschätzt [59]. Ziel dieser Studie war es daher, den Einfluss des Zeitpunktes der Blutentnahme (vor vs. nach der Entfernung des Primärtumors) (1) auf die CTC-Detektionsrate zu untersuchen und (2) die klassischen prädiktiven Merkmale wie HR- und HER2-Status des Primärtumors und der prä- bzw. postoperativ detektierten CTC zu vergleichen.

Methoden:

Blutproben von 209 primären Mammakarzinompatientinnen wurden präoperativ und 2-3 Tage (48-72 h, median 58h) nach der Entfernung des Primärtumors auf CTC hin untersucht. Der Nachweis erfolgte mittels des Multiplex-RT-PCR-basierten, kommerziell erhältlichen AdnaTestBreast-CancerDetect-Assay, basierend auf der Detektion von drei tumorassoziierten Transkripten: GA733-2, Muc1 und HER-2. Bei CTC-positiven Patientinnen wurde der Östrogenrezeptor(ER)-Status und Progesteronrezeptor(PR)-Status der detektierten Zellen bestimmt. Die Phänotypisierung der Primärtumore (ER, PR, HER2) erfolgte mittels klassischer Immunhistochemie.

Ergebnisse:

Bei 43 von 209 Mammakarzinompatientinnen (21%) konnten CTC prä- und/oder postoperativ nachgewiesen werden. Die präoperative Detektionsrate unterschied sich mit 12% nicht signifikant von der postoperativen Detektionsrate von 16% (p=0,264). Die prä- und postoperative Positivitätsraten korrelieren nicht miteinander (p=0,169). Der präoperative CTC-Nachweis korrelierte signifikant mit einem Nodalbefall, während die postoperative CTC-

Positivität mit keinem der klinisch-pathologischen Faktoren assoziiert war. Die meisten CTC wiesen einen tripel-negativen Phänotyp auf (24 von 43 Patientinnen, 56%), gefolgt von HER2-positiv/HR-negativ bei 10 von 43 Patientinnen (23%) und HR-positiv bei 9 von 43 Patientinnen (21%). Im Gegensatz dazu hatten 41 der 43 CTC-positiven Patientinnen (95%) HR-positive Primärtumore. Insgesamt war der Phänotyp des Primärtumors und der CTC nur bei 8 der 43 Patientinnen (19%) konkordant, während der prä- und postoperative CTC-Phänotyp in 88% der Fälle konkordant waren.

Schlussfolgerung:

Die Entfernung des Primärtumors hat die CTC-Positivitätsrate im Blut in diesem großen Patientenkollektiv nicht beeinflusst. Des Weiteren konnte eine hohe phänotypische Diskrepanz zwischen dem Primärtumor und den CTC in Bezug auf klassische prädiktive Faktoren wie HR- und HER2-Status gezeigt werden, wobei der häufigste CTC-Phänotyp tripel-negativ war. Gleichzeitig waren der prä- und postoperative Phänotyp der CTC in der meisten Fällen konkordant. Diese Ergebnisse lassen schlussfolgern, dass die nachgewiesenen CTC im Blut möglicherweise aus den sog. sekundären Kompartimenten wie Knochenmark stammen und sich unabhängig vom Primärtumor entwickeln. Mit Hilfe der phänotypischen Charakterisierung dieser Zellen können möglicherweise potenzielle therapeutische Angriffspunkte identifiziert werden.

3.3 Prognostische Relevanz der spontanen und Chemotherapie-induzierten Apoptose von disseminierten Tumorzellen beim primären Mammakarzinom

Krawczyk N, Hartkopf A, Banys M, Meier-Stiegen F, Staebler A, Wallwiener M, Röhm C, Hoffmann J, Hahn M, Fehm T. Prognostic relevance of induced and spontaneous apoptosis of disseminated tumor cells in primary breast cancer patients. BMC Cancer. 2014 Jun 3;14:394. doi: 10.1186/1471-2407-14-394. PMID: 24894702; PMCID: PMC4055221.

Einleitung:

Ein Ungleichgewicht zwischen der Zellproliferation und dem programmierten Zelltod, meist als Folge genetischer Veränderungen bestimmter Onkogene, spielt eine wichtige Rolle im Prozess der Tumorentstehung und des Tumorwachstums [60-64]. Studien zufolge weisen hoch proliferative Mammakarzinome mit aggressiver Tumorbiologie und schlechter Prognose auch eine hohe Rate an apoptotischen Tumorzellen auf [62, 65, 66]. Gleichzeitig induzieren die meisten Zytostatika die Tumor-Apoptose zur Erreichung der Tumorregression [67, 68]. Der Nachweis apoptotischer DTC als Ausdruck des Therapieansprechens bei Mammakarzinompatientinnen unter neoadjuvanter Chemotherapie wurde von unserer Gruppe bereits demonstriert [69]. Das Ziel dieser Studie war es, die Inzidenz (1) und prognostische Signifikanz (2) der apoptotischen DTC bei primär operierten und neoadjuvant behandelten Mammakarzinompatientinnen zu vergleichen.

Methoden:

383 primäre Mammakarzinompatientinnen mit positivem Knochenmarkstatus wurden in diese Studie eingeschlossen. 85 Patientinnen wurden primär operiert und 298 Patientinnen erhielten eine neoadjuvante Chemotherapie. Die Knochenmarkspunktion erfolgte jeweils intraoperativ. Bei diesen DTC-positiven Patientinnen erfolgte zusätzlich der immunzytochemische Nachweis von apoptotischen Tumorzellen. Hierfür wurde der M30-Antikörper angewandt, der ein nach der Spaltung von Zytokeratin 18 während der frühen Apoptose entstehendes Neoepitop detektiert. Der Nachweis von M30-positiven DTC wurde mit klinisch-pathologischen Faktoren in beiden Gruppen verglichen. Das mediane Follow-up betrug 44 Monate (10-88 Monate).

Ergebnisse:

82 von 298 primär operierten Patientinnen (27%) und 41 der 85 neoadjuvant behandelten Patientinnen (48%) hatten mindestens eine zusätzliche apoptotische (M30-positive) Tumorzelle im Knochenmark. In der neoadjuvant behandelten Gruppe hatten die M30-positiven Patientinnen signifikant seltener ein Rezidiv erlitten, verglichen mit den Patientinnen ohne M30-postive Zellen im Knochenmark (7% vs. 23% der Ereignisse, p=0,049). Im Gegensatz dazu war der Nachweis apoptotischer Tumorzellen bei primär operierten Patientinnen mit signifikant schlechterem Gesamtüberleben assoziiert (5% vs. 12% der Ereignisse, p=0,008).

Schlussfolgerung:

Apoptotische DTC können im Knochenmark von Mammakarzinompatientinnen vor und nach der systemischen Behandlung nachgewiesen werden. Die Apoptose der Tumorzellen nach der Neoadjuvanz kann durch Zytostatika induziert werden. Dies erklärt die unterschiedliche prognostische Bedeutung der spontanen und Chemotherapie-induzierten Apoptose bei diesen zwei Patientinnengruppen.

3.4 Androgenrezeptor-Status von zirkulierenden Tumorzellen beim metastasierten Mammakarzinom

Krawczyk N, Neubacher M, Meier-Stiegen F, Neubauer H, Niederacher D, Ruckhäberle E, Mohrmann S, Hoffmann J, Kaleta T, Banys-Paluchowski M, Reinecke P, Esposito I, Janni W, Fehm T. Determination of the androgen receptor status of circulating tumour cells in metastatic breast cancer patients. BMC Cancer. 2019 Nov 12;19(1):1101. doi: 10.1186/s12885-019-6323-8. PMID: 31718606; PMCID: PMC6852746.

Einleitung:

Die prognostische Relevanz der CTC im peripheren Blut von Patientinnen mit metastasiertem Mammakarzinom (MBC; metastatic breast cancer) wurde in mehreren Studien demonstriert [70]. Der Nachweis von mehr als 5 Zellen in 7.5ml Blut ist mit einem signifikant schlechteren Überleben assoziiert und die Dynamik der Tumorzellzahl während der Behandlung spiegelt das Therapieansprechen in diesem Kollektiv wider [47]. Die Frage nach der prädiktiven Bedeutung des CTC-Phänotyps und der klinischen Rolle der CTC-basierten Therapieentscheidungen wird im Rahmen der DETECT Studien untersucht [71]. Die neusten Ergebnisse der DETECT III Analyse haben gezeigt, dass eine sich nach dem CTC-Phänotyp orientierende, zielgerichtete Behandlung zu einem signifikanten Überlebensvorteil bei MBC Patientinnen führen kann [54]. Die Suche nach neuen CTC-assoziierten prädiktiven Markern spielt in diesem Kontext eine zunehmend wichtige Rolle.

Der Androgen-Rezeptor (AR) wird bei über 70% der HR-positiven und bei bis zu 45% der tripel-negativen Mammakarzinome exprimiert. Die zielgerichtete anti-AR Therapie beim Mammakarzinom wird derzeit im Rahmen von zahlreichen Studien untersucht und zeigt im metastasiertem AR-positiven Kollektiv vielversprechende Ansprechraten von bis zu 25% [23, 24]. Da die Bestimmung des AR-Status beim Mammakarzinom nicht routinemäßig erfolgt, ist dieser meistens unbekannt, sodass es bei therapeutischen Überlegungen in der metastasierten Situation einer AR-Neubestimmung im archivierten Primärtumorgewebe oder einer gezielten Metastasenbiopsie bedarf. Vor diesem Hintergrund können die CTC im peripheren Blut als sog. flüssige Biopsie (*Engl. liquid biopsy*) dienen und eine attraktive, nicht invasive Alternative zur klassischen Metastasenbiopsie darstellen. Das Ziel dieser Untersuchung war es, den AR-Status von CTC im Kollektiv metastasierter Mammakarzinompatientinnen zu untersuchen.

Methoden:

Peripheres Blut von metastasierten Mammakarzinompatientinnen wurde im Rahmen der DETECT-Untersuchung auf CTC gescreent. In der Gruppe der CTC-positiven Patientinnen wurde eine zusätzliche Blutprobe mittels CellSearch® Profile Kit immunmagnetisch angereichert und auf einen Objektträger übertragen. Eine Immunfluoreszenztripelfärbung zum Nachweis von AR-positiven CTC wurde im Rahmen der Arbeit etabliert. Prostatakarzinom-Zelllinien LNCaP und DU145 dienten entsprechend als Positiv- und Negativkontrollen. Basierend auf der positiven Färbung von DAPI und Panzytokeratin (CK) sowie der negativen CD45-Färbung wurden die kernhaltigen, epithelialen Zellen identifiziert und die Leukozyten ausgeschlossen.

Ergebnisse:

Eine Immunfluoreszenztripelfärbung wurde im Rahmen der Analyse erfolgreich etabliert. Blutproben von 67 metastasierten Mammakarzinompatientinnen, die im Rahmen der DETECT Studie CTC-positiv gescreent wurden, wurden in diese Subanalyse eingeschlossen. Bei 37 der 67 Patientinnen (56%) wurde zumindest eine CTC in der zusätzlichen Blutprobe nachgewiesen. Bei 16 von 37 Proben (43%) konnten AR-positive CTC detektiert werden. Bei 8 von 25 Patientinnen (32%), die mehr als eine CTC im Blut hatten, wurden sowohl AR-positive als auch AR-negative CTCs nachgewiesen. Es wurde eine heterogene, zytoplasmatische und nukleäre AR-lokalisation beobachtet

Schlussfolgerungen:

AR positive CTC können im peripheren Blut von metastasierten Mammakarzinompatientinnen nachgewiesen werden. Die prädiktive Bedeutung dieser Zellen, insbesondere der heterogenen Zelllokalisation des AR, soll in weiteren Studien etabliert werden.

3.5 SOX2 Status der disseminierten Tumorzellen im Knochenmark und des Primärtumors bei neoadjuvant behandelten Mammakarzinompatientinnen

Krawczyk N, Janowski K, Banys-Paluchowski M, Staebler A, Neubauer H, Meisner C, Hartkopf A, Brucker S, Wallwiener D, Fehm T. The SOX2 Status of Disseminated Tumor Cells in Breast Cancer Patients Treated With Neoadjuvant Chemotherapy. Anticancer Res. 2021 Jun;41(6):2849-2858. doi: 10.21873/anticanres.15066. PMID: 34083275

Einleitung:

Der Nachweis von DTC im Knochenmark von primären Mammakarzinompatientinnen nach Abschluss der systemischen Behandlung ist mit einer ungünstigen Prognose assoziiert [55]. Diese persistierenden Tumorzellen gelten als Surrogatmarker der minimal residualen Krebserkrankung. Gleichzeitig entwickeln nicht alle DTC-positiven Patientinnen einen Krankheitsrückfall, sodass vermutet wird, dass nur eine kleine Subpopulation dieser Zellen ein Metastasierungspotenzial besitzt. In diesem Kontext werden die potenziellen Stammzelleigenschaften der DTC diskutiert. Studien zufolge können Brustkrebszellen einen Prozess der sog. epithelial-mesenchymalen Transition durchlaufen und Stammzelleigenschaften gewinnen [72-75]. Dieses Phänomen wurde auch bei DTC primärer Mammakarinompatientinnen beschrieben, allerdings wurde dies nur selten bei Zustand nach neoadjuvanter Chemotherapie untersucht [76-78]. Das Ziel dieser Studie war die Bestimmung der Expression eines Stammzellmarkers SOX2 auf disseminierten Tumorzellen und im Primärtumorgewebe von nicht metastasierten Mammakarzinompatientinnen, die eine neoadjuvante Chemotherapie erhalten haben.

Methoden:

170 DTC-positive Mammakarzinompatientinnen nach neoadjuvanter Chemotherapie wurden in diese Studie eingeschlossen. Ein zusätzlicher KM-Objektträger wurde auf SOX2-positive DTC hin untersucht. Eine Immunfluoreszenzdoppelfärbung zur Bestimmung des SOX2-Status wurde mit Hilfe der kolorektalen Karzinom-Zelllinie HT29 etabliert. Die SOX2-Expression im Primärtumorgewebe wurde immunhistochemisch mit Hilfe desselben Antikörpers bestimmt.

Ergebnisse:

Der SOX2-Status der DTC konnte bei 62 Patientinnen bestimmt werden, wobei 20 dieser Patientinnen (36%) mindestens eine SOX2-positive Zelle im KM aufwiesen. Der SOX2-Status der DTC war mit keinem der klinisch-pathologischen Faktoren assoziiert. Die meisten der

untersuchten Primärtumore zeigten keine SOX2-Expression (30 von 38 (79%) und 18 von 27 (67%) entsprechend vor und nach der Neoadjuvanz). Bei 36% der Patientinnen (5 von 14) mit SOX2-negativem Tumor konnten persistierenden SOX2-positive DTC im KM nachgewiesen werden.

Schlussfolgerungen:

DTC, die im Knochenmark von Mammakarzinompatientinnen nach Abschluss der neoadjuvanten Chemotherapie persistieren, können Stammzelleigenschaften aufweisen. SOX2-positive DTC wurden auch bei Patientinnen mit SOX2-negativen Primärtumoren nachgewiesen, was auf eine potenzielle Unabhängigkeit dieser Tumorzellpopulationen hinweist. Im Rahmen weiterer Studien soll der Stammzellcharakter der DTC auf genomischer Ebene bestätigt sowie die klinische Relevanz dieser Zellpopulation untersucht werden.

3.6 Invasives Mammakarzinom mit neuroendokriner Differenzierung: eine Einzelzentrumanalyse der klinischen und prognostischen Eigenschaften

Krawczyk Natalia, Röwer Rowena, Anlauf Martin, Muntanjohl Caja, Baldus Stephan Ernst, Neumann Monika, Banys-Paluchowski Maggie, Otten Sabine, Luczak Katharina, Ruckhäberle Eugen, Mohrmann Svjetlana, Hoffmann Jürgen, Kaleta Thomas, Jaeger Bernadette, Esposito Irene, Fehm Tanja Invasive breast carcinoma with neuroendocrine differentiation: a single-center analysis of clinical features and prognosis Geburtshilfe Frauenheilkd. 2021 (accepted)

Einleitung:

Das invasive Mammakarzinom mit neuroendokriner Differenzierung (MC-NE) stellt eine sehr seltene Form des Mammakarzinoms mit einer in der Literatur berichteten Prävalenz zwischen 0,1 und 20% aller Mammaneoplasien dar [27]. Diese Diskrepanz ist einerseits durch die häufigen Änderungen in der Definition dieser heterogenen Läsionsgruppe in den letzten zwei Dekaden verursacht, andererseits dadurch, dass die Bestimmung der neuroendokrinen Marker nicht zur Routinediagnostik beim Mammakarzinom gehört [28]. Daher können die korrekte Diagnosestellung und Erörterung der genauen Prävalenz sowie die Identifizierung von spezifischen klinischen Eigenschaften und potenziellen prädiktiven Faktoren bei diesen Tumoren eine Herausforderung darstellen. Das Ziel dieser retrospektiven Studie war (1) die Untersuchung der klinischen Charakteristika und Therapiestrategien (2), die Untersuchung der prognostischen Bedeutung der neuroendokrinen Differenzierung in einer großen Kohorte von Patientinnen mit BC-NE sowie (3) der Vergleich unserer Ergebnisse mit den *bis dato* publizierten Daten.

Methoden:

27 Patientinnen mit BC-NE wurden in diese Untersuchung eingeschlossen. 21 Fälle wurden durch eine systematische immunhistochemische Re-Evaluation von 465 Mammakarzinompräparaten mit Hilfe der spezifischen neuroendokrinen Marker Chromogranin A und Synaptophysin identifiziert. Dies resultierte in einer Prävalenz von 4,5%. Die weiteren 6 Fälle wurden anhand der Aktenanalyse identifiziert und die BC-NE Diagnose wurde pathologisch bestätigt.

Ergebnisse:

Das mediane Alter bei der Diagnosestellung betrug 61 Jahre. 70 % der Patientinnen hatten T2-4 Tumore und 37% waren nodal-positiv. Der häufigste immunhistochemische Tumortyp war HR-positiv/HER2-negativ (85%). Bei 93% der Tumore konnte die Expression von

Synaptophysin und bei 48% von Chromogranin A in jeweils mehr als 50 % der Tumorzellen nachgewiesen werden. Der Somatostatin Rezeptor Typ 2a (SSTR2A) Status war bei 12 der 24 untersuchten Tumoren (50%) positiv. Neuroendokrin-spezifische Diagnostik erfolgte in 5 Fällen, während die Therapie mit Somatostatin-Analoga bei 2 Patientinnen verabreicht wurde. Das 5-Jahre Gesamtüberleben betrug in dem untersuchten Kollektiv 70%.

Schlussfolgerungen:

Mammakarzinom mit neuroendokriner Differenzierung ist meistens HR-positiv und HER2-negativ, die Diagnose wird, verglichen mit dem konventionellen Mammakarzinom, in einem höheren TNM-Stadium gestellt. BC-NE zeigte in unserer Analyse sowie in mehreren retrospektiven Studien eine ungünstige Prognose, wobei bei vielen dieser Studien (inklusive unserer Untersuchung) kein direkter Vergleich mit dem konventionellen Mammakarzinom erfolgte. Ein relevanter Teil dieser Tumore exprimiert den SSTR-2A Rezeptor, was die spezifische SSTR-basierte Diagnostik erlaubt und in individuellen Situationen eine SSTR-zielgerichtete Therapie ermöglicht.

4. Zusammenfassung

Das invasive Mammakarzinom stellt bereits in den Frühstadien eine systemische Erkrankung dar, die neben dem lokalen therapeutischen Ansatz in der Regel einer systemischen Behandlung bedarf. In diesem Kontext steht die Identifikation von neuen prognostischen sowie potenziellen prädiktiven Markern im Vordergrund der heutigen Mammakarzinomforschung mit dem Ziel, die Risikopatientinnen zu identifizieren und die Behandlung möglichst individuell (zielgerichtet) und effizient zu gestalten. Das übergeordnete Ziel der dargestellten wissenschaftlichen Arbeiten war es, die neuen phänotypischen Biomarker im Primärtumorgewebe sowie peripheren Blut und Knochenmark von Mammakarzinom-patientinnen zu untersuchen.

Phänotypische Charakterisierung der DTC und CTC beim primären Mammakarzinom

Die Tumorzelldissemination beim Mammakarzinom findet bereits früh im Krankheitsverlauf statt und gilt als potenzieller Ursprung der späteren Metastasierung. Der Nachweis von DTC im Knochenmark nicht metastasierter Mammakarzinompatientinnen zum Zeitpunkt der Erstdiagnose [79] sowie nach Abschluss der adjuvanten Chemotherapie [55] ist mit einem verkürzten Überleben assoziiert. Vor diesem Hintergrund stellt die Eradikation dieser Zellen ein potenzielles Ziel der zukünftigen adjuvanten Therapieansätze dar. In der ersten im Rahmen dieser Arbeit vorgestellten Originalpublikation wurde der HER2 Status von persistierenden DTC im KM untersucht und mit dem HER2 Status des Primärtumors verglichen. Hier konnte gezeigt werden, dass die disseminierten Tumorzellen, die nach Abschluss der adjuvanten zytotoxischen Therapie persistieren, einen positiven HER2-Status aufweisen können, auch wenn der Primärtumor HER2-negativ ist. Ähnliche phänotypische Unterschiede bezüglich des HER2-Status zwischen DTC und Primärtumor wurden auch zum Zeitpunkt der Erstdiagnose beobachtet [57, 58]. Dieses Phänomen wird einerseits als Ausdruck des aggressiveren Phänotyps der DTC im Vergleich mit dem Primärtumor angesehen, andererseits als Ausdruck der bekannten intratumoralen Heterogenität [80]. Des Weiteren lässt sich durch diese Ergebnisse das Versagen der adjuvanten, sich nach dem Phänotyp des Primarius richtenden Therapien erklären.

In einer weiteren hier vorgestellten Originalarbeit wurde die SOX2-Expression auf DTC im Knochenmark von neoadjuvant behandelten Mammakarzinompatientinnen untersucht. Der SOX2 ist ein Transkriptionsfaktor und embryonaler Stammzellmarker, dessen onkogene Rolle bei verschiedenen Neoplasien, inklusive Mammakarzinom, beschrieben wurde [81-85]. Im Rahmen unserer Analyse konnte zum ersten Mal gezeigt werden, dass die nach

neoadjuvanter Chemotherapie persistierenden DTC diesen Stammzellmarker exprimieren können. Auch bezüglich der SOX2-Expression konnte eine häufige phänotypische Diskrepanz zwischen DTC und Primärtumor beobachtet werden. In 41% der untersuchten Fälle waren der SOX2 Status der DTC und des Primärtumors unterschiedlich, wobei SOX2-positive DTC bei Patientinnen mit SOX2-negativem Primärtumor häufiger nachgewiesen werden konnten. Die Hypothese, dass eine bestimmte DTC-Subpopulation Stammzelleigenschaften aufweisen kann und diese Tumorstammzellen für die spätere Metastasierung verantwortlich sind, wurde in den letzten Jahren immer intensiver postuliert. Das Erlangen mesenchymaler bzw. stammzellartiger Eigenschaften im Rahmen der sog. epithelial-mesenchymalen Transition kann einerseits das hohe Metastasierungspotenzial dieser Zellen erklären, andererseits ihre Fähigkeit, sich der zytotoxischen Therapie zu entziehen und in einem ruhenden/schlafenden Zustand zu persistieren [59, 86].

Neben der lange bekannten klinischen Relevanz von DTC im Knochenmark wurde die prognostische Bedeutung der CTC im peripheren Blut von nicht metastasierten Mammakarzinompatientinnen mittlerweile in zahlreichen Studien bestätigt [40]. Im Rahmen einer weiteren Publikation wurde der Einfluss der Operation auf die Detektionsrate und den Phänotyp von CTC unter Berücksichtigung des Phänotyps des Primarius untersucht. Der beobachtete fehlende Einfluss der Operation auf die CTC-Detektionsrate lässt vermuten, dass die nachgewiesenen CTC möglicherweise über die Operation hinaus aus den sog. sekundären Kompartimenten in das Blut gelangen können. Des Weiteren wurde beobachtet, dass sich die Expression von wichtigen prädiktiven Faktoren (ER, PR und HER2) zwischen CTC und Primärtumor unterscheiden kann. Während die Mehrzahl der in die Analyse eingeschlossenen Primärtumore HR-positiv war, wiesen die meisten detektierten CTC den tripel-negativen Status auf. Diese Ergebnisse reflektieren erneut den postulierten aggressiveren Charakter von zirkulierenden Tumorzellen. Darüber hinaus weist die in allen drei Arbeiten beobachtete phänotypische Diskrepanz zwischen DTC/CTC und dem Primärtumor möglicherweise darauf hin, dass sich die Zellen der MRD unabhängig von den Zellen des Primarius entwickeln können. Diese Beobachtung steht im Einklang mit dem von Klein et al. postulierten Modell der parallelen Tumorprogression [4].

In der weiteren Originalarbeit wurde die klinische Bedeutung der spontanen und der durch Chemotherapie induzierten Apoptose von DTC untersucht. Es konnte gezeigt werden, dass M30-positive, apoptotische DTC unterschiedliche prognostische Bedeutung haben, je nachdem, ob es sich um therapienaive oder zytotoxisch behandelte Patientinnen handelt. Der Nachweis apoptotischer DTC bei therapienaiven Patientinnen war mit einem signifikant schlechteren Gesamtüberleben assoziiert, während Patientinnen mit M30-positiven DTC nach Neoadjuvanz signifikant seltener ein Krankheitsrezidiv erlitten haben. Diese Ergebnisse lassen

vermuten, dass die DTC-Apoptose in der neoadjuvanten Gruppe möglicherweise durch die Chemotherapie induziert wird und somit das Therapieansprechen widerspiegelt. Im Gegensatz dazu spiegelt die spontane Apoptose von DTC vermutlich die Aggressivität des Tumors wider. Die Assoziation zwischen hohen Raten an apoptotischen Tumorzellen und erhöhtem Proliferationsindex sowie hohem Grading wurde zuvor im Primärtumorgewebe von Mammakarzinompatientinnen beschrieben [62, 65, 66]. Es konnte hier im großen Kollektiv von primären Mammakarzinompatientinnen demonstriert werden, dass dieses Phänomen auch in DTC beobachtet werden kann.

Phänotypische Charakterisierung der CTC bei metastasiertem Mammakarzinom

Die Rolle der zirkulierenden Tumorzellen bei metastasiertem Mammakarzinom sowohl als prognostischer Faktor als auch als ein Tool zum Therapiemonitoring konnte in den letzten Jahren in zahlreichen Studien bestätigt werden [87]. Eine prädiktive Bedeutung dieser Zellen und die klinische Rolle einer CTC-basierten Therapieentscheidung bleibt derzeit noch unklar, wobei die neusten Ergebnisse der DETECT III Studie zeigen, dass eine auf dem CTC-Phänotyp basierte Therapie das Überleben der MBC Patientinnen verbessern kann [54]. Im Rahmen einer hier vorgestellten Originalarbeit ist der Androgenrezeptorstatus als mögliches Therapietarget auf den CTCs von MBC Patientinnen untersucht worden. Nach Etablierung einer Immunfluoreszenz-Dreifachfärbung konnte gezeigt werden, dass AR-positive Tumorzellen im peripheren Blut nachgewiesen werden können. Die antiandrogene Therapie wird derzeit in zahlreichen Studien untersucht, sodass diese Arbeit einen möglichen Ansatzpunkt in Hinblick auf eine potenzielle CTC-basierte Therapiestratifizierung in der Zukunft darstellt. Des Weiteren konnte eine heterogene Lokalisation des Androgenrezeptors auf CTCs beobachtet werden, ein Phänomen, das auch in CTCs bei metastasiertem Prostatakarzinom beschrieben wurde [88]. Welche Rolle diese heterogene Lokalisation in der Prädiktion des Therapieansprechens spielt und ob der AR-Status der CTCs als prädiktiver therapeutischer Marker dienen kann, muss in weiteren größeren Studien evaluiert werden.

Phänotypische Charakterisierung des Primärtumors

In der letzten im Rahmen dieser Arbeit vorgestellten Originalpublikation wurde die klinische Rolle der neuroendokrinen (NE) Differenzierung beim Mammakarzinom analysiert. In der durchgeführten Untersuchung einer eigenen Patientenkohorte mit dieser extrem seltenen Tumorentität und im Vergleich mit den bis dato publizierten Studien konnte die prognostische Rolle der NE-Differenzierung bestätigt werden. Darüber hinaus wurde gezeigt, dass eine systematische immunhistochemische Bestimmung neuroendokriner Marker zur Erhöhung der

Detektionsrate dieses Mammakarzinom-Subtyps beiträgt. In unserer Untersuchung haben 50% der analysierten Patientinnen eine Expression von Somatostatin-Rezeptor 2A aufgewiesen. Die SSTR-2A Expression bei Mammakarzinom mit NE-Differenzierung wurde *bis dato* nur in einer weiteren Studie untersucht [32]. Wir konnten hier zeigen, dass die SSTR-2A-Positivität eine spezifische Diagnostik und, in ausgewählten Fällen, eine zielgerichtete Therapie mit Somatostatin-Analoga ermöglicht.

Mit den dieser kumulativen Habilitationsschrift zugrundeliegenden Originalarbeiten konnte das bisherige Wissen über phänotypische und blutbasierte Biomarker beim Mammakarzinom erweitert werden. Es konnten vielfältige phänotypische Eigenschaften und deren prognostische Relevanz bei disseminierten und zirkulierenden Tumorzellen beim primären Mammakarzinom gezeigt werden. Diese Zellen zeigen oft einen HER2-positiven oder tripel-negativen Status und können Stammzellmarker exprimieren als möglichen Ausdruck ihrer Aggressivität. Darüber hinaus konnten neue potenzielle phänotypische Targets auf zirkulierenden Tumorzellen im metastasierten Setting identifiziert werden. Schließlich konnte der neuroendokrine Phänotyp des Primärtumors als potenzieller prognostischer Marker beim Mammakarzinom bestätigt werden.

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6. Abkürzungsverzeichnis

AR	Androgenrezeptor
CTC	zirkulierende Tumorzelle(n)
DTC	disseminierte Tumorzelle(n)
ER	Östrogenrezeptor
HR	Hormonrezeptor
HER2	human epidermal growth factor receptor 2
MBC	metastasiertes Mammakarzinom
MRD	minimale residuale Krebserkrankung
PR	Progesteronrezeptor
SOX2	SEX determining Region Y (SRY)-BOX2
SSTR-2A	Somatostatinrezeptor 2A

7. Schriftenverzeichnis

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8. Lebenslauf

PERSÖNLICHE DATEN

Geburtsdatum / -ort:	17.02.1981, Breslau/Polen
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UNIVERSITÄRER UND BERUFLICHER WERDEGANG

04’2018	Schwerpunkt „Spezielle Geburtshilfe und Perinatalmedizin“
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04’2016 - dato	Oberärztin, Universitätsfrauenklinik Düsseldorf
09‘2014 - 03’2016	Fachärztin für Frauenheilkunde und Geburtshilfe
04’2013 - 08’2014	Assistenzärztin, Universitätsfrauenklinik Düsseldorf
08’2009 - 03’2013	Assistenzärztin, Frauenklinik Klinikum Bremen-Mitte
2005 – 2009	Medizinstudium, Eberhard-Karls-Universität Tübingen
02.07.2009	Approbation
26.06.2009	Staatsexamen (Note: sehr gut)
2008 – 2009	Praktisches Jahr Gynäkologie: Universitätsfrauenklinik Tübingen Innere Medizin: Universidad Autónoma de Madrid, Spanien Chirurgie: Chirurgische Universitätsklinik Tübingen
2000 – 2007	Medizinstudium, Universität Breslau, Polen
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Seit 04’2013	Universitätsfrauenklinik Düsseldorf Wissenschaftliche Mitarbeiterin in der Arbeitsgruppe: Translationale Gynäkoonkologie

12`2010 PROMOTION

Universitätsfrauenklinik Tübingen; Betreuerin: Frau Professor T. Fehm
Thema: „Bestimmung des ERα-Status von disseminierten Tumorzellen im Knochenmark bei Patientinnen mit primärem Mammakarzinom“
Note: magna cum laude

PREISE / STIPENDIEN FÖRDERUNGEN

2020	Förderprogramm für Habilitandinnen im Fachbereich Medizin “Chancen ergreifen - Forschung und Familie fördern”
2018	GebFra Preis 2018 für den Übersichtsartikel Endometriosis-associated Malignancy Geburtshilfe und Frauenheilkunde 2016
2012	Posterpreis der AGO-Kommission TRAFO, 4. Wissenschaftliches Symposium der Kommission Translationale Forschung, Bergisch-Gladbach (Koautorin)
2007	TOP TEN Poster, 2. Wissenschaftliches Symposium der Kommission Translationale Forschung der AG Gynäkologische Onkologie, Bergisch Gladbach
2005 – 2006	Stipendium der Stiftung ROTARIER

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10. Vollständige Publikationen zur kumulativen Habilitation

Nach freundlicher Genehmigung der Verlage

ANTICANCER RESEARCH *29*: 4019-4024 (2009)

HER2 Status on Persistent Disseminated Tumor Cells after Adjuvant Therapy May Differ from Initial HER2 Status on Primary Tumor

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Abstract. *Background: Persistence of disseminated tumor cells (DTCs) is observed in 10 to 15% of breast cancer patients and is associated with poor prognosis. These patients might benefit from secondary adjuvant targeted therapy. The aim of this study was to assess HER2 status of persistent DTCs to determine whether the use of HER2-targeted agents might be a therapeutic option in patients with tumor cell persistence. Patients and Methods: Bone marrow was obtained from 85 primary breast cancer patients intraoperatively and after completion of systemic treatment (median follow-up of 13 months; range: 6-30 months). Immunofluorescence double staining was used for identification of cytokeratin-positive, HER2-positive cells. Results: A total of 31 out of 85 (36%) patients had DTCs preoperatively. Out of 85 (16%) patients, 14 were DTC positive after completion of surgery and adjuvant cytotoxic therapy. Five of these patients had HER2-positive DTCs, however, the corresponding tumor was HER2 positive in only one case. The remaining nine patients with HER2-negative DTCs had HER2-negative primary tumors. Conclusion: HER2-positive DTCs can be detected in patients with HER2-negative tumors, even after adjuvant therapy. Such patients may benefit from (secondary) HER2-targeted therapy in an adjuvant setting.*

Persistence of disseminated tumor cells (DTCs) in bone marrow after completion of surgery and adjuvant chemotherapy can be observed in 10 to 15% of breast cancer patients. As demonstrated by the European pooled analysis, tumor cell persistence is associated with poor clinical outcome (1). Therefore, the targeted elimination of these cells might be a highly promising therapeutic strategy to improve prognosis in these patients. While the accurate nature of DTCs is still under research, attempts have been made over the last decade to characterize these cells with regard to both pheno- and genotype.

Human epidermal growth factor receptor 2 (HER2), one of the tyrosine kinase erb-B receptors, belongs to the most relevant predictive factors in breast cancer (2). HER2-positive tumors tend to be of a more aggressive biological behavior (2). The clinical role of HER2 gained in importance after the introduction of the anti-HER2 monoclonal antibody trastuzumab and other novel anticancer agents such as pertuzumab and lapatinib (3, 4). HER2 is overexpressed in 20 to 30% of primary breast cancer patients and this group may benefit from targeted therapy (5, 6). The indication for molecular antibody therapy is based on HER2 overexpression or gene amplification in the primary tumor. However, several studies suggested that disseminated and circulating breast cancer cells may acquire positive HER2 status independently of the primary tumor and may become a potential target for a molecular antibody therapy in an adjuvant or metastatic setting (7-10). Abandonment of a targeted therapy in this patient collective could thus result in unintentional undertreatment.

The aims of our study were (a) to assess how many patients have persistent DTCs after completion of adjuvant therapy and (b) to evaluate the HER2 status of DTCs themselves at the time of diagnosis and after the therapy to determine whether trastuzumab and other molecular targeted agents might be a therapeutic option for the elimination of persistent DTCs.

Patients and Methods

Patients. After written informed consent, bone marrow samples were obtained intraoperatively from 85 primary breast cancer patients who were treated at the Department of Gynecology and Obstetrics (University Hospital Tuebingen, Germany) from 2001 until 2006. The patients were then treated with adjuvant

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Key Words: Breast cancer, persistent disseminated tumor cells, trastuzumab, targeted therapy, HER2 status.

chemotherapy, hormone therapy, or both based on current St. Gallen recommendations and national treatment guidelines (www.ago-online.de). After a median follow-up of 13 months (range: 6-30 months), a second bone marrow aspiration was performed. Clinical data of patients are shown in Table I. Only two patients received trastuzumab as part of their adjuvant therapy, since the majority of patients in this study were diagnosed with breast cancer before trastuzumab was considered as a standard therapy in HER2-positive breast cancer.

Detection and characterization of DTCs. Ten to twenty ml of bone marrow were prepared by centrifugation on a Ficoll-Hypaque density gradient (1.077 g/ml; Biochrom, Germany) followed by lysis of red blood cells with lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2). Mononuclear cells (10^6 MNC/spot) were then cytospun onto a glass slide (Hettich cytocentrifuge, Germany) and air-dried overnight at room temperature. A double immunofluorescence staining procedure was performed for the detection of HER2-positive tumor cells. Slides were fixed with 0.5% neutral-buffered formalin for 10 minutes and washed twice in phosphate-buffered saline (PBS). Nonspecific antibody binding was blocked using 10% Goat Serum Normal (DAKO, Denmark) in PBS for 30 minutes. Primary rabbit HER2-antibody CB11 (1:100; Biogenex, CA, USA) was applied for 30 minutes, followed by incubation with secondary goat anti-rabbit antibody, labeled with Texas Red (1:100; Vector Laboratories, CA, USA) for 30 minutes. Subsequently, directly labeled FITC-C11 mouse monoclonal antibody against pan-cytokeratin (CK) (1:100; Sigma, MI, USA) was added and slides were incubated for 30 minutes. Nuclei were counterstained with 4'6-diamidino-2-phenylindole in mounting media (Vector Laboratories). The breast cancer cell line SKBR3 (ATCC®-Nr. HTB-30, American type culture collection, Manassas, VA, USA) was used as a positive control for cytokeratin and HER2 staining and the cell line MCF7 (ATCC®-Nr. HTB-22D, American type culture collection) as negative control for HER2 staining. Leukocytes of a healthy volunteer served as a negative control for both. The microscopic analysis of slides was performed by two independent investigators (NK and TF). Evaluation for the presence of tumor cells was carried out using a computerized fluorescence microscope (Axiophot; Zeiss, Germany). A single-pass filter for individual fluorochromes (FITC, Texas Red or DAPI) and a dual-pass filter for FITC/Texas Red were used to screen for HER2-positive tumor cells. Only cells with moderately or strongly stained membrane were considered HER2-positive. Criteria for the identification of single HER2-positive DTCs by immunofluorescence are described in more detail by Meng *et al*. (11) and Solomayer *et al*. (8).

Staining of the primary tumor. Core cut biopsies or surgically resected specimens were analyzed immunohistochemically for expression of HER2 protein. Sections 3-5 μm-thick of formalin-fixed, paraffin-embedded tissue were stained using commercially available ABC kit (Vectastain; Vector Laboratories). Sections were incubated with primary polyclonal HER2 antibody (clone A 0485) diluted 1:200 in Tris-HCl according to the manufacturer's instruction (HERCEPTM test; Dako, Glostrup, Denmark). Color development was achieved with 3,3'-diaminobenzidine (DAB). Slides were counterstained with hematoxylin and mounted for examination. HER2 expression was evaluated using HercepTest criteria. The HER2 score was based on a 0 to +3 scale. Tumors with a score of +2/+3 were considered HER2 positive. In case of a score of +2, fluorescence *in situ* hybridization was performed to determine HER2 amplification using the Pathvysion™ kit (HER2/NEU) (Vysis, Downers Grove, IL, USA). The scoring conditions followed the recommendations given by the manufacturer.

Statistical analysis. Chi-squared test was used to examine the association between clinicopathological factors and detection of CK and/or HER2-positive tumor cells. Statistical analysis was performed using SPSS (Version 16, SPSS GmbH Software, Germany) considering *p*-values less than 0.05 to be statistically significant.

Results

Bone marrow status before adjuvant treatment. Bone marrow aspirates from 85 patients were analyzed for the presence of persistent DTCs. The first bone marrow aspiration was performed at the time of surgery and the second after a median follow-up of 13 months (range: 6-30 months). The identification of DTCs was based on cytokeratin positivity and morphological criteria according to the Consensus Recommendations for Standardized Tumor Cell Detection (12). Typical morphology of a representative cytokeratin-positive tumor cell is shown in Figure 1. In 31 (36%) patients, DTCs were detected in bone marrow. The number of DTCs ranged from 1 to 5 cells per patient (2×10^6 mononuclear cells). A statistical correlation was found between intraoperative DTC-positive bone marrow status and negative estrogen receptor status of primary tumor but not with any other of the established prognostic markers, including the HER2 status of the primary tumor (Table I).

In 8 out of 31 (26%) cases with DTC-positive bone marrow status, HER2 positivity of DTCs was observed. Nevertheless, only one of these 8 patients demonstrated an HER2 overexpressing primary tumor. Four out of 23 (17%) patients with HER2-negative DTCs showed HER2 positivity of their primary lesion. The comparison of HER2 status between primary tumor and DTCs is shown in Table II.

Heterogeneity of HER2 expression in DTCs. In 23 out of 31 (74%) patients with detectable DTCs in bone marrow at the time of primary diagnosis, only HER2-negative DTCs were found. In the remaining 8 (26%) patients, HER2-overexpressing cells were observed. In 3 out of 7 (43%) patients with more than one tumor cell in bone marrow there was heterogeneity of HER2 expression (Figure 2).

HER2 status of DTCs after completion of adjuvant therapy. Persistent DTCs after therapy were found in 14 out of 85 (16%) patients. No statistical correlation between DTC-positive bone marrow status after treatment and any of the established prognostic markers including the HER2 status of the primary tumor was observed (Table I).

Five out of 14 (36%) patients with tumor cell persistence had HER2-positive DTCs. However, the corresponding tumor was HER2 positive in only one case. All nine patients with HER2-negative DTCs also had HER2-negative primary tumors (Table II). Nevertheless, the percentage of patients with HER2-positive DTCs was higher during follow-up (5 out of 14; 36%) compared to the intraoperative time point (8 out of 31 patients; 26%).

Five patients with HER2-positive tumors showed DTCs at the time of first diagnosis. Since trastuzumab was not part of the standard treatment before 2005, these patients did not receive HER2-targeted therapy. After completion of adjuvant treatment, only one of these patients had persistent tumor cells which were HER2 positive, although initially only HER2-negative DTCs had been detected.

Table I. *Clinical data of patients.*

		Before treatment		After treatment	
	N	BM positive (%)	*p*-value	BM positive (%)	*p*-value
Total	85	31 (36)		14 (16)	
Menopausal status					
Premenopausal	28	13 (46)	n.s.	10 (17)	n.s.
Postmenopausal	57	18 (32)		4 (14)	
Tumor size					
pT1	53	21 (37)	n.s.	8 (15)	n.s.
pT2-4	32	10 (31)		6 (19)	
Nodal status					
Node negative	58	24 (41)	n.s.	8 (16)	n.s.
Node positive	26	7 (27)		6 (23)	
Histology					
Ductal	59	22 (37)	n.s.	8 (14)	n.s.
Lobular	19	7 (37)		3 (16)	
Other	7	2 (29)		3 (43)	
Grading					
I	3	1 (33)	n.s.	1 (33)	n.s.
II	63	22 (35)		11 (17)	
III	17	7 (41)		0 (0)	
ER status					
Negative	20	11 (55)	0.05	5 (25)	n.s.
Positive	65	20 (31)		9 (14)	
PR status					
Negative	29	13 (45)	n.s.	7 (24)	n.s.
Positive	56	18 (32)		7 (12)	
HER2					
Negative (0/+1)	71	27 (38)	n.s.	13 (19)	n.s.
Positive (+2/+3)	14	4 (29)		1 (7)	
Systemic adjuvant therapy					
Chemotherapy	19	11 (58)	n.s.	4 (21)	n.s.
Endocrine therapy	25	8 (32)		3 (12)	
Both	41	12 (29)		7 (17)	

n.s.: Not significant; BM positive: presence of disseminated tumor cells in bone marrow; ER: estrogen receptor; PR: progesterone receptor.

Discussion

Tumor cell persistence. Recent studies suggest that a selected subgroup of patients may benefit from extended adjuvant treatment. Of all validated prognostic factors, monitoring of minimal residual disease is the only one available after the primary tumor has been removed. A large pooled analysis demonstrated a strong negative impact of persistent DTCs on both disease-free and overall survival (1). Thus, follow-up bone marrow screening might help to identify patients who are most likely to develop disease recurrence and would potentially benefit from a secondary adjuvant therapy. While the exact biological nature of DTCs is still to be further investigated, various study groups examined their phenotype with regard to novel therapeutic agents. Since biological factors of DTCs differ from those of the primary tumor, their correct assessment may improve our understanding of the natural history of breast cancer and enable us to optimize therapy regimens. HER2 status has proven to be one of the most important predictive factors in breast cancer and is routinely determined in primary tumor. Targeted therapy drugs, such as trastuzumab, pertuzumab and lapatinib, were introduced into breast cancer treatment in both metastatic and adjuvant settings.

HER2 status of DTCs does not reflect the HER2 status of the primary tumor. Several aspects of HER2 status on DTCs must be considered. Firstly, DTCs reflect only a subpopulation of cancer cells from primary tumor. This selected group of cells seem to feature factors commonly associated with poorer clinical outcome, such as negative hormonal status and up-regulation of urokinase-type plasminogen activator receptor (13, 14). Additionally, HER2-positive tumor cells have an enhanced extravasative potential, and thus a growth and survival advantage, and can therefore be encountered more frequently in bone marrow or blood (15). As a result, the HER2 status of DTCs or other metastatic sites does not necessarily reflect the HER2 status of the primary tumor. In our patient group, HER2-positive tumor cells were detected in the bone marrow of seven patients despite their having HER2-negative primary tumors. This finding is consistent with previous publications (7, 8, 16-19) (Table III). As the indication for trastuzumab-targeted therapy is based on HER2 overexpression or gene amplification of the primary tumor, a subgroup of patients with HER2-positive DTCs but HER2-negative tumors is not eligible for this treatment. However, several studies have demonstrated that trastuzumab-based therapy is able to eliminate HER2-positive circulating tumor cells (11, 20, 21). Whether the indication for trastuzumab treatment in an adjuvant setting should be extended to patients with HER2-positive DTCs regardless of primary tumor status must be further evaluated.

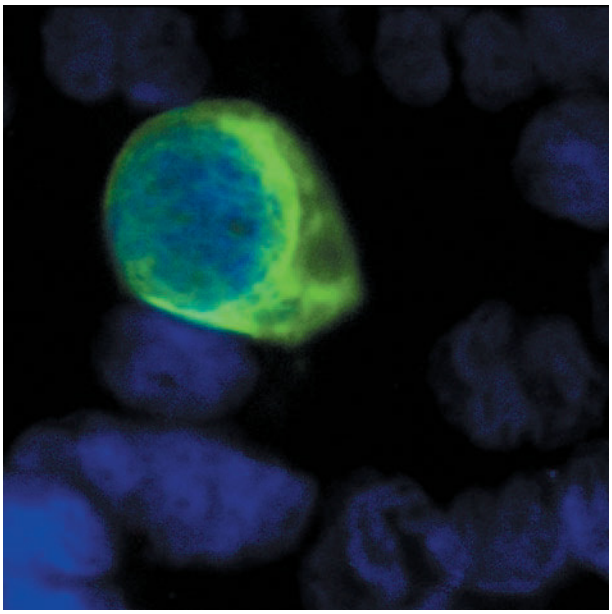

Figure 1. *Typical cytomorphology (nuclear size clearly enlarged, high nuclear to cytoplasmic ratio) and immunophenotype (irregular cytoplasmic staining for cytokeratin, cytokeratin filaments can be seen) of a representative DTC from a breast cancer patient. Tumor cell is stained with an anti-CK-fluorescein isothiocyanate (green) antibody (×40 oil immersion objective).*

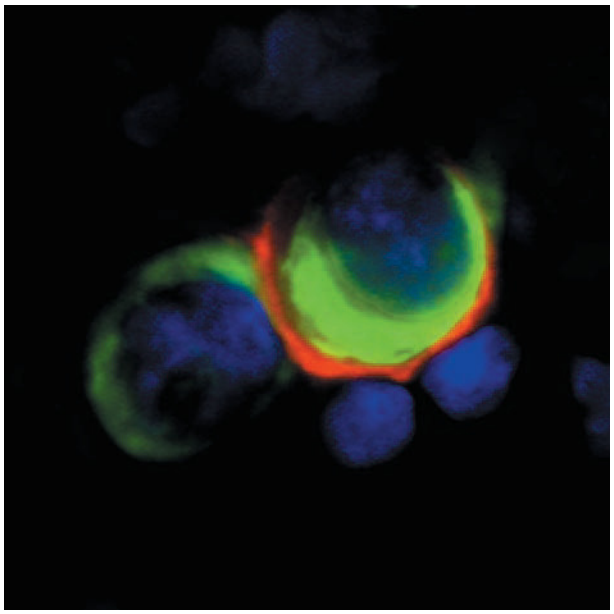

Figure 2. *Heterogeneity of HER2 expression on DTCs from a primary breast cancer patient. Tumor cells were stained with an anti-CK-fluorescein isothiocyanate (green) and anti-HER2 detected by a secondary Texas Red labeled goat anti-rabbit (red) antibodies. Nuclei are stained blue with DAPI (×40 oil immersion objective). Cluster of HER2-negative and HER2-positive DTCs from a breast cancer patient.*

HER2 overexpression can be acquired during dissemination and progression. Furthermore, persistent DTCs may acquire a more aggressive phenotype in the course of the disease. We observed an increase of patients with HER2-positive DTCs (26% at the time of surgery, 33% after follow-up). As shown before, conventional adjuvant chemotherapy fails to eliminate DTCs from bone marrow (22). One major reason for this inefficiency is the dormant state of DTCs with a small proliferation index (23). However, at some point, single tumor cells might increase their metabolism, leaving the dormant state, and thus cause subsequent metastasis.

Recently, Jückstock *et al*. presented preliminary results of an interventional post-adjuvant trastuzumab-based pilot trial (24). Twelve asymptomatic breast cancer patients with persistent HER2-positive DTCs received trastuzumab. All patients completed chemotherapy at least 6 months prior to entering the study. Trastuzumab treatment was able to eradicate DTCs in seven of these patients. Another interesting approach was proposed by Bernhard *et al*. Autologous HER2-specific T-lymphocytes were transferred to a patient with metastatic HER2-positive breast cancer. This experimental treatment was able to eliminate HER2-overexpressing tumor cells from the bone marrow, but did not penetrate into solid metastases (25). However, elimination of minimal residual disease may not have a direct impact on survival outcome. Whether patients with persistent DTCs actually benefit clinically from additional targeted therapy strategies will have to be evaluated in further prospective randomized studies.

Disease monitoring and response to therapy. The acquisition of more aggressive genomic aberrations, such as HER2 amplification, may indicate tumor progression and play a role in the metastatic cascade (11). This patient group might benefit from additional targeted therapy. This underlines the need for re-evaluation and monitoring of DTC status in the course of the disease (10). In contrast to tissue evaluation with regard to HER2 overexpression – a single event – monitoring minimal residual disease gives an opportunity for real-time insight into disease progression. The persistence of HER2-positive circulating tumor cells after completion of adjuvant chemotherapy was shown to be linked to poor clinical outcome (16).

Conclusion

Concluding, in the present report, we were able to show that the HER2 status on persistent DTCs differs not only from that of the primary tumor, but also from the intraoperative

Table II. *Course of DTCs in primary breast cancer during treatment and follow-up.*

HER2 status		N	DTC-positive after treatment	DTC HER2 status after treatment	
Primary tumor	DTCs before treatment			Positive	Negative
Negative	Negative	19	3	0	3
	Positive	7	2	1	1
	No DTCs	44	8	3	5
Positive	Negative	4	1	1	0
	Positive	1	0	-	-
	No DTCs	10	0	-	-
Total		85	14	5	9

Table III. *Correlation of HER2 status between primary tumor and DTCs.*

Study group	N	HER2 positivity (%)		Concordance
		Primary tumor	DTCs	
Present[a]	31	5 (16)	8 (26)	64%
Present[b]	14	1 (7)	5 (36)	71%
Jueckstock et al. (24)[b]	129	34 (26)	49 (38)	68%
Apostolaki et al. (16)[a]	212	24 (11)	52 (25)[c]	69%
Solomayer et al. (8)[a]	45	14 (29)	20 (44)	63%
Becker et al. (9)[a]	105	26 (25)	22 (21)	77%
Braun et al. (19)[a]	24	7 (29)	15 (63)	58%
Meng et al. (11)[d]	33	15 (46)	11 (33)	88%

[a]DTC at the time of diagnosis; [b]persistent DTC after completion of therapy; [c]CTC in peripheral blood, not DTC in bone marrow; [d]breast cancer patients at recurrence.

DTC status. HER2 positivity may be acquired during dissemination and tumor progression. Whether the indication for targeted trastuzumab treatment should be based on both primary tumor and DTC status must be further evaluated.

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PRECLINICAL STUDY

The influence of removal of primary tumor on incidence and phenotype of circulating tumor cells in primary breast cancer

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Abstract Recent studies have shown that the detection of circulating tumor cells (CTC) pre and postoperatively in the peripheral blood of primary breast cancer patients may be an indicator for poor survival. This study aimed to investigate the influence of removal of the primary tumor on incidence and phenotype of circulating tumor cells in primary breast cancer. 209 primary breast cancer patients could be included into this analysis. Blood sampling was performed both pre and postoperatively. The blood specimens were immunomagnetically enriched using AdnaTest BreastCancerSelect within 4 h after blood withdrawal, followed by RNA isolation and subsequent gene expression analysis by reverse transcription and multiplex PCR using AdnaTest BreastCancerDetect. Three breast cancer-associated tumor markers and two hormone receptor genes were amplified: GA733-2, Muc-1, Her-2, ER, PR. In addition, bone marrow (BM) status was intraoperatively determined. Forty-three of 209 patients (21%) had pre and/or postoperatively circulating tumor cells. The positivity rates after surgery were higher but did not differ significantly (12% pre and 16% postoperatively, $P = 0.264$). Disseminated tumor cells in BM were seen in 32 of 209 cases (15%). Patients with positive BM status had significantly higher CTC positivity rates both pre and postoperatively compared to those with negative BM status. The most common CTC phenotype was triple negative (24 patients) followed by HER2+/ER−/PR− subtype (10) and ER and/or PR positive (9). Interestingly, 41 of 43 primary tumors (95%) were ER and PR positive. Removal of the primary tumor did not alter the phenotype of CTC. Surgery does not significantly influence the tumor cell load in the blood stream. CTC phenotype before and after the surgery generally remains identical but may differ from that of the primary tumor.

Keywords Breast cancer · Circulating tumor cells · Surgery · Tumor cell phenotype

Abbreviations

BM	Bone marrow
CK	Cytokeratin
CTC	Circulating tumor cell
DTC	Disseminated tumor cell
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
MRD	Minimal residual disease
PR	Progesterone receptor

Introduction

The presence of disseminated tumor cells (DTC) in bone marrow (BM) is a common phenomenon seen in 30–40% of primary breast cancer patients. As demonstrated by a large, pooled analysis of BM specimens from more than 4,700 patients, DTC presence at the time of diagnosis is an

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independent prognostic factor [1]. In addition, it has been shown that tumor cells are able to survive chemotherapy [2] and that their persistence is strongly associated with poor outcome [3]. However, one limitation of BM biopsy is its invasiveness. Since BM sampling is not well tolerated by many patients, detection of circulating tumor cells (CTC) in the blood might be an ideal option. CTC are routinely detected, depending on stage of the disease and methodology, in 10–80% breast cancer patients. Conclusive data on the clinical relevance of CTC is pending; nevertheless, recent studies have shown a prognostic potential of CTC in primary and metastatic breast cancer patients [4, 5].

While biological significance of DTC is generally accepted, circulating tumor cells are considered by many to be an epiphenomenon of primary tumors. According to this model, single tumor cells are shed mainly by the primary tumor, and their phenotype necessarily reflects the tumor's characteristics. Since the estimated half-life of CTC does not exceed 2.4 h, a rapid decrease in CTC incidence after tumor removal would be expected [6]. We aimed to verify this hypothetical model by examining changes in CTC incidence before and after surgery. It is also assumed that the ability to disseminate and persist is not common for all tumor cells but rather requires a selected subpopulation of cancer cells with particular expression profiles. Thus, CTC phenotype was determined before and after removal of the primary tumor. In addition, since DTC represent an established and stable marker for minimal residual disease (MRD) [7], CTC detection was compared to bone marrow status.

Materials and methods

Patients

This prospective analysis was performed at the Department of Obstetrics and Gynecology in University Hospital Tuebingen, Germany. In total, 209 primary breast cancer patients (pT1-4, pN0-2, M0) were recruited between 2006 and 2009. Patients' characteristics at the time of diagnosis are shown in Table 1. None of the patients underwent neoadjuvant systemic therapy. All specimens were obtained after written informed consent and collected using protocols approved by the institutional review board (114/2006A). The phenotype of the primary tumor was routinely assessed by immunohistochemical staining in the Department of Pathology, University of Tuebingen. Staining protocols are described in detail elsewhere [8].

Sampling of blood

5 ml EDTA blood samples were collected before surgery and 2 or 3 days after surgery (48–72 h, median 58 h) and stored at 4°C until further examination. The samples were processed immediately or not later than 4 h after blood withdrawal. Blood samples were analyzed for CTC with the AdnaTest BreastCancer (AdnaGen AG, Langenhagen, Germany) which enables the molecular detection of tumor cells via epithelial and tumor-associated antigens. The system was described in detail previously [9]. Blood samples were incubated with a ready-to-use antibody mixture commercialized as AdnaTest BreastCancerSelect according to the manufacturer's instructions. Two antibodies against the epithelial antigen MUC1 and one antibody against the epithelial glycoprotein GA 733-2 (EpCAM) were conjugated to magnetic beads (Dynabeads) for the labeling of tumor cells in peripheral blood [10]. The labeled cells were extracted by a magnetic particle concentrator (MPC).

Multiplex RT-PCR

mRNA isolation from lysed, enriched cells was performed according to the manufacturer's instructions with the Dynabeads mRNA DIRECT Micro Kit (Dynal Biotech GmbH, Hamburg, Germany) that is included in the AdnaTest BreastCancerDetect. Reverse transcription resulted in cDNA, which served as a template for tumor cell detection and characterization by multiplex RT-PCR. Sensiscript Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany) was used for the reverse transcription because of its high sensitivity (recommended for amounts of <50 ng RNA) in combination with oligo(dT) coupled Dynabeads of the mRNA DIRECT™ Micro Kit (Dynal Biotech GmbH) [11]. cDNA was synthesized in a thermocycler under the following conditions. Reverse transcription was performed at 37°C for 60 min followed by 3 min at 93°C for inactivation of the reaction. The resulting cDNA was stored at −20°C until further use. The analysis of tumor-associated mRNA isolated from CTC was performed in a multiplex PCR for three tumor-associated transcripts: HER2, MUC1, and GA 733-2. The primer sets for the estrogen and progesterone receptor (ER, PR) were provided from Adnagen AG (Langenhagen, Germany). ER and PR were detected on CTC after the preparation of cDNA and according to the manufacturer's instructions. PCR was performed with the HotStarTaq Master Mix (QIAGEN GmbH). β-Actin was used as internal PCR positive control. The thermal profile used for the nested RT-PCR was as follows. After a 15 min denaturation at 95°C, 37 cycles of PCR were carried out by denaturation at 94°C for 30 s, annealing/extension at 60°C for 30 s and elongation for 30 s at 72°C. Subsequently, termination of the reaction was carried out at 72°C for 5 min followed by storage of the samples at 4°C. The primers generated fragments of the following sizes: GA 733-2: 395 base pairs

Table 1 Clinical data of patients

	n $N = 209$	CTC positive preoperative (%)	*P* value	CTC positive postoperative (%)	*P* value	DTC positive (%)	*P* value
Total	209	26 (12)		34 (16)		32 (15)	
Menopausal status			n.s.		n.s.		n.s.
Premenopausal	42	3 (7)		5 (12)		5 (12)	
Postmenopausal	167	23 (14)		29 (17)		27 (16)	
Tumor size			n.s.		n.s.		n.s.
pT1	149	16 (11)		23 (15)		20 (13)	
pT2-4	60	10 (17)		11 (18)		12 (20)	
Nodal status			0.031		n.s.		n.s.
Negative	155	15 (10)		21(13)		22 (14)	
Positive	52	11 (21)		13(25)		9 (17)	
Histology			n.s.		n.s.		n.s.
Ductal	153	22 (14)		26 (17)		22 (14)	
Lobular	34	3 (9)		5 (15)		5 (15)	
Others	21	1 (5)		3 (14)		4 (19)	
Grading			n.s.		n.s.		0.016
I/II	158	17 (11)		24 (15)		19 (12)	
III	49	9 (18)		10 (20)		13 (27)	
ER status			n.s.		n.s.		n.s.
Negative	33	2 (6)		4 (12)		6 (18)	
Positive	176	24 (14)		30 (17)		26 (15)	
PR status			n.s.		n.s.		n.s.
Negative	40	6 (15)		7 (17)		10 (25)	
Positive	169	20 (12)		27 (16)		22 (13)	
HER2 status			n.s.		n.s.		n.s.
Negative	192	25 (13)		31 (16)		27 (15)	
Positive	10	1 (10)		3 (30)		3 (27)	
BM status			0.026		0.005		
DTC	32	8 (25)		11 (34)			
No DTC	177	18 (10)		23 (13)			

BM bone marrow, *CTC* circulating tumor cells, *DTC* disseminated tumor cells, *ER* estrogen receptor, *PR* progesterone receptor

(bp), MUC1: 293 bp, HER2: 270 bp, PR: 270 bp, ER: 305 bp, and β-actin: 114 bp. Visualization of the PCR fragments was carried out with a 2100 Bioanalyzer using the DNA 1000 LabChips (Agilent Technologies) and the Expert Software Package (version B.02.03.SI307).

Evaluation of data established for CTC

The test was considered positive if a PCR fragment of at least one tumor-associated transcript was clearly detected. Using the software package for evaluation of the data on the Agilent 2100 Bioanalyzer, peaks with a concentration of >0.15 ng/µl were positive for the transcripts GA733-2, MUC1, and HER2. Peaks with a concentration of >0.60 ng/µl were considered positive for the ER transcript. The PR expression was considered positive when the transcript was detected without applying any cut-off.

Detection of disseminated tumor cells in bone marrow

DTC detection was performed as described in detail previously [12]. 10–20 ml bone marrow (BM) was aspirated from the iliac crest into syringes containing heparin anticoagulant under general anesthesia using Jamshidi's technique. Tumor cell isolation and detection was performed based on the recommendations for standardized tumor cell detection [13]. Samples were separated by density centrifugation using Ficoll (density 1,077 g/ml, Biochrom, Germany). Mononuclear cells were collected from the interphase layer and were spun down onto a glass slide (Hettich cytocentrifuge, Germany) (10^6 MNC/spot). For detection of cytokeratin-positive (CK) tumor cells, slides were fixed in 4% neutral buffered formalin for 10 min and rinsed in PBS. Automatic immunostaining was performed on the DAKO Autostainer using the monoclonal mouse A45-B/B3 antibody (Micromet, Germany) and the DAKO-

APAAP detection kit (DakoCytomation, Denmark) according to the manufacturer's instructions. The A45-B/B3 antibody is directed against common cytokeratin epitopes including the CK heterodimers 8/18 and 8/19. The malignant breast cell line MCF-7 was used as a positive control. For each patient 2×10^6 cells were analyzed on two slides. Analysis was performed on the Automated Cellular Imaging System (ACIS, ChromaVision Medical Systems, San Juan, Capistrano, CA).

Statistical analysis

Chi-squared test and Fisher's exact test were used to evaluate the relationship between CTC and clinicopathological factors. The McNemar test was used to compare the relationship of CTC positivity before and after surgery. Statistical analysis was performed by SPSS, version 11.5 (SPSS Inc., Chicago, IL, USA). *P* values below 0.05 were considered statistically significant.

Results

Patients' characteristics

A total of 209 patients were included in the study. Seventy-one per cent (149 of 209) of these patients had T1 tumors and 75% (155 of 209) were node negative. The most common immunohistochemical phenotype, based on the ER, PR and HER2 expression of primary tumor was ER and/or PR positive (190 patients, 90%) followed by triple-negative (ER−/PR−/HER2−; 15 patients, 7%) and HER2+/ER−/PR− (4 patients, 2%). Clinical data are shown in detail in Table 1. The distribution of patients is summarized in a Recommendations for Tumor Marker Prognostic Studies (REMARK) diagram [14] (Fig. 1).

Incidence of CTC before and after surgical therapy

In 43 of 209 patients (21%), circulating tumor cells were detected before and/or after the surgery. In 26 (12%) patients CTC were detected preoperatively. 34 patients (16%) were CTC positive postoperatively (Table 2). CTC positivity before surgery was not associated with CTC positivity after surgery ($P = 0.169$). Nine patients (4%) were only preoperatively positive for CTC, 17 (8%) only postoperatively and 17 (8%) patients both pre and postoperatively. Pre and postoperative positivity rates did not differ significantly (12% and 16%, respectively, $P > 0.05$). Preoperative CTC status correlated with nodal involvement ($P = 0.031$) but not with other clinicopathological factors. No correlation could be observed between postoperative CTC status and established prognostic factors.

Incidence of DTC in BM

In 32 of 209 patients (15%), DTC in bone marrow could be detected at the time of surgery. The timepoint of CTC positivity was associated with BM status ($P = 0.011$). Bone marrow positivity was highest in patients with CTC before and after surgery (41%; 7 out of 17) followed by patients with postoperatively detected CTC (24%; 4 out of

Table 2 Positivity rates before and after surgery

	Postoperative CTC pos.	Postoperative CTC neg.	Total
Preoperative CTC pos.	17 (8%)	9 (4%)	26 (12%)
Preoperative CTC neg.	17 (8%)	166 (79%)	183 (88%)
Total	34 (16%)	175 (84%)	209 (100%)

CTC positive at least once: 43 (21%)

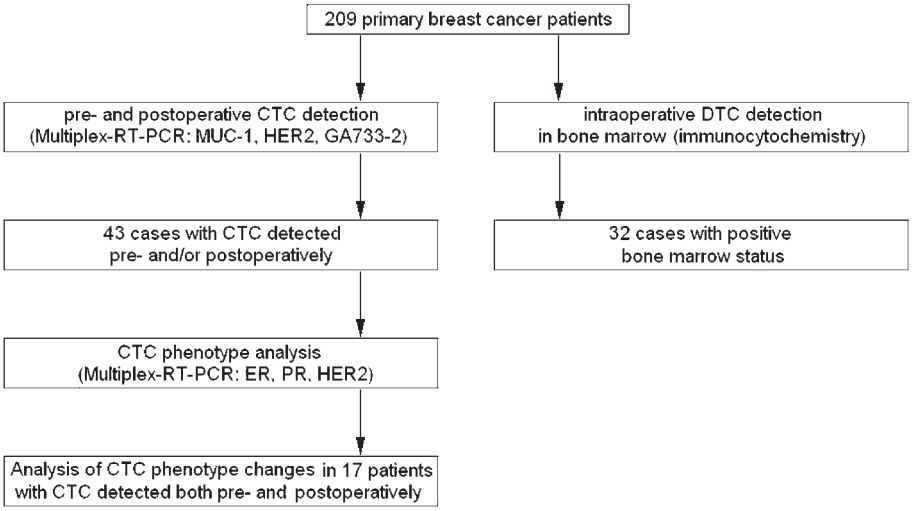

Fig. 1 Patient distribution diagram according to the Recommendations for Tumor Marker Prognostic Studies (REMARK)

17). In contrast, DTC could only be detected in 12 and 11% of patients with no CTC (20 of 166) and CTC positivity before surgery (1 of 9), respectively ($P = 0.01$). However, this observation is prone to sampling error due to small sample size.

Comparison of expression profiles of primary tumor and CTC

In 43 patients, CTC were detected in at least one time point. In these patients an additional gene expression profile with regard to ER and PR status was determined. CTC were considered ER/PR/HER2 positive if the corresponding receptor was positive before and/or after surgery. In 8 of 43 (19%) patients, CTC were ER and/or PR positive while 16 (37%) patients had HER2 positive CTC. The most common CTC phenotype was triple negative (ER/PR/HER2 negative; 24 patients; 56%) followed by HER2+/ER−/PR− (10 patients; 23%) and ER and/or PR positive (9 patients; 21%).

Primary tumors were in 3 cases (7%) HER2 positive and in 41 cases (95%) hormone receptor positive (ER and/or PR). However, 33 of these 41 patients (80%) presented with ER/PR negative CTC. One patient had a triple-negative tumor and HER2 positive CTC. In one case, CTC were ER/PR positive while the primary tumor was HER2 positive but ER/PR negative (Table 3).

Table 3 Comparison of expression profiles of primary tumor and CTC

	CTC expression profile			
	Triple neg. *n*	HER2+/ER−/PR− *n*	ER/PR pos. *n*	Total
Primary tumor expression profile				
Triple neg.	–	1	–	1
HER2+/ER−/PR−	–	–	1	1
ER/PR pos.	24	9	8	41
Total	24	10	9	43

Table 4 CTC expression profile pre and postoperatively

CTC expression profile	Postoperative *N*			
	Triple neg.	HER2+/ER−/PR−	ER/PR pos.	Total
Preoperative *N*				
Triple neg.	9	1	–	10
HER2+/ER−/PR−	1	3	–	4
ER/PR pos.	–	–	3	3
Total	10	4	3	17

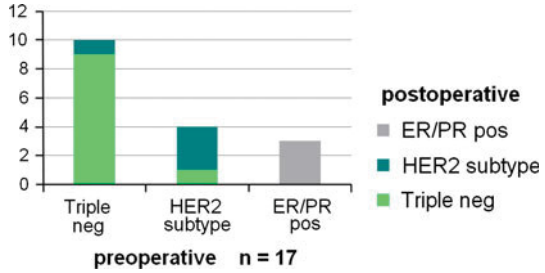

Fig. 2 The phenotype of CTC before and after removal of the primary tumor

Changes in expression profiles of CTC

In 17 of 209 patients (8%) CTC were detected both pre and postoperatively. Table 4 shows expression profiles of CTC before and after surgery. A switch in HER2 status of CTC was observed in only two patients while the ER/PR status remained unchanged in all 17 patients (Fig. 2).

Discussion

Incidence of CTC before and after surgery

With a total of 209 patients included in analysis, this is the largest study to date to examine the fate of CTC after removal of a primary tumor. Our results demonstrate no significant change in CTC incidence before and after surgical therapy (12 vs. 16%, respectively), and similar findings have been reported by others previously in smaller cohorts [15], [16]. In a group of 41 patients, Biggers et al. reported a CTC incidence of 24% preoperatively and 30% 2 weeks after surgery [17]. Recently, Sandri et al. detected CTC using the CellSearch system in 29% breast cancer patients preoperatively and in 30% patients 5 days post-surgery [16]. Remarkably, CTC status changed in 40% of all patients. Further, Krag et al. showed, using an exceedingly sensitive assay (95% incidence at the time of diagnosis) that CTC incidence in breast cancer falls rapidly following surgery [18]. Nevertheless, as in our study, a considerable proportion of patients failed to eliminate CTC after surgery, the incidence reaching a stable plateau (approx. 30%) at 48–72 h postoperatively. Interestingly, despite a high variation in preoperative CTC incidence, most of the studies observed 15–30% positivity rate after surgery [19, 20]. Table 5 summarizes the data regarding changes in CTC load due to surgery.

Tumor cell persistence

Our data suggest that tumor cells may persist in secondary sites, independent of the primary tumor even in early stages

Table 5 A summary of other studies investigating the CTC changes due to removal of the primary tumor

Author	Year	*N*	Primary tumor	Method	CTC positivity rate preoperatively (%)	Time of obtaining the postoperative blood sample	CTC positivity rate postoperatively (%)	Conversion rate (%)
Sandri et al. [16]	2010	56	Breast cancer	CellSearch	16/56 (29%)	5 days postop	14/47 (30%)	19/47 (40%)
						30 days postop[a]	8/27 (30%)	
Weitz et al. [15]	1998	58	Colorectal cancer	RT-PCR	15/58 (26%)	24 h postop	12/58 (21%)	9/58 (15%)
Bessa et al. [20]	2001	50	Colorectal cancer	RT-PCR	35/50 (70%)	24 h postop	23/50 (46%)	16/50 (32%)
Sawabata et al. [19]	2007	9	Non small cell lung cancer	CellSearch	1/9 (11%)	24 h postop	3/9 (33%)	2/9 (22%)
Biggers et al. [17]	2009	41	Breast cancer	CellSearch	10/41 (24%)	14 days postop	9/30 (30%)	–
Krag et al. [18]	1999	21	Breast cancer	ICC	18/19 (95%)s	2 h postop	15/18 (83%)	–
						4 h postop	14/19 (74%)	
						8 h postop	9/16 (56%)	
						12 h postop	7/14 (50%)	
						24 h postop	10/19 (53%)	
						48 h postop	6/19 (32%)	
						7 days postop	3/18 (17%)	
						14 days postop	6/20 (30%)	
Our study	2011	209	Breast cancer	RT-PCR (Adnagen)	26/209 (12%)	48–72 h postop	34/209 (16%)	26/209 (12%)

[a] In case of positivity of the preoperative and/or postoperative sample at 5 days, another sample was taken after 30 days

ICC immunocytochemistry, *RT-PCR* reverse transcription polymerase chain reaction

of the disease. A comprehensive analysis of the half-life of tumor cells was presented by Meng et al. [6]. Based on serial measurements, the CTC in patients whose primary tumor was just removed had a half-life measured in 1–2.4 h. Since the estimated half-life of CTC is very short, and a major fraction of detectable cells are apoptotic, CTC must be continuously replenished [6, 21]. However, in the postoperative setting the primary tumor as the CTC releasing source is no longer present, so the existence of an alternative actively self-renewing population ("cancer stem cells") must be assumed (Fig. 3). In contrast to the "classic" model of metastatic cascade, where any tumor cell may be the source of subsequent cell growth and metastasis, the stem cell model postulates a critical role of progenitor cell population in cancer development and progression [22]. These cells with highly tumorigenic properties (e.g. capability of self-renewal and differentiation) are supposed to play a major part in several malignancies, such as breast and gastrointestinal cancer, retinoblastoma and ovarian cancer [23, 24]. It has been shown that CTC and DTC in primary breast cancer display stem cell features, such as ALDH1 positivity or presence of CD44 and absence of CD24 [22, 25, 26].

Correlation of CTC with bone marrow status

Tumor cell dissemination in bone marrow of primary breast cancer patients is considered a surrogate marker for minimal residual disease, whereas the role of circulating tumor cells in the peripheral blood of these patients remains less well known. In our study, we compared the incidence of tumor cells in both compartments in order to evaluate the potential of CTC to act as an additional marker for MRD. Our data show a significant correlation between DTC and CTC status both pre and postoperatively (25 vs. 10%, 34 vs. 13%, respectively; Table 1). However, the concordance between CTC positivity and bone marrow status was higher for postoperative than preoperative CTC status (*P* value 0.005 and 0.026, respectively). This discrepancy may be the result of blood contamination with tumor cells shed from primary lesion in preoperative setting. Hypothetically, such cells represent an epiphenomenon of primary tumor and are no longer present after the surgery, since the source of the spread has been removed. Postoperatively detected persistent CTC seem to be strongly linked to DTC in bone marrow. This suggests a possible role of these cells as a marker for MRD, since all

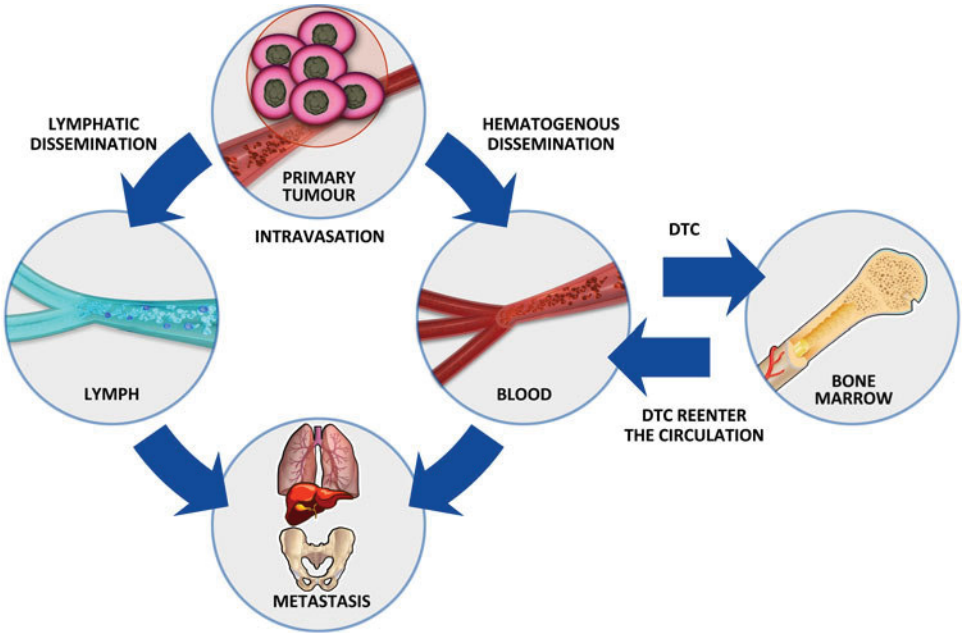

Fig. 3 Theoretical model of metastatic spread. Single tumor cells from the primary tumor enter blood circulation at the earliest stage of the disease (hematogenous dissemination) and migrate into secondary homing site, e.g. bone marrow. This favorable environment allows for their long-year persistence in dormant state and accumulation of further genetic changes. Subsequently tumor cells re-enter the circulation and disperse into different tertiary sites

patients in our study had no evidence of metastatic disease [27]. Significant correlation between tumor cell detection in both compartments after the removal of the primary tumor was also reported by other authors [28, 29].

Comparison of expression profile of CTC and primary tumor

Single tumor cells in secondary sites seem to be a perfect surrogate marker for minimal residual disease. Besides mere detection of CTC/DTC, the need to determine their expression profile is becoming increasingly important, as they are targets of all adjuvant treatment strategies. However, the indication to and the choice of adequate therapy are based exclusively on the properties of primary tumor [2]. Various authors reported a discrepancy between tumor cells from primary tumors and those in secondary sites, such as blood and bone marrow, with regard to ER and HER2 status [30, 31, 32, 33]. This might be relevant to clinicians when selecting patients for targeted therapy, as patients with HER2-negative tumors but HER2-positive MRD are not eligible for HER2-based treatment. However, these patients might benefit from such therapy [34, 35]. Inversely, the loss of ER-positivity in disseminated or circulating tumor cells may explain the failure of endocrine therapy in a subset of ER-positive patients [33]. In our patient group, the expression profiles of CTC differed significantly from corresponding primary tumors. These observations suggest a more complex relationship between primary tumor and minimal residual disease, with substantial differences both at the genomic and phenotypic level. CTC may reflect only a subpopulation of cancer cells from the primary tumor with the ability to disseminate and as such feature factors commonly associated with poorer clinical outcome [33, 36]. In addition, isolated tumor cells are able to acquire new genomic alteration in course of disease progression [35]. In our study, expression profiles of primary tumors were assessed by immunohistochemistry while the phenotype of CTC was determined by RT-PCR. Clearly, it would be desirable that the same method is used to determine phenotype of both primary tumor and CTC. However, the test used in our study does not allow further immunohistochemical analysis.

Changes in expression profiles of CTC

Conceptual models of tumor growth have been developed to improve our understanding of the natural history of cancer. Two basal models of cancer behavior and progression were discussed recently by Klein [37]. In the linear progression model, primary tumor undergoes local progression, accumulating genetic and morphological abnormalities, until fully malignant cells are able to disseminate from the primary site and subsequently seed metastasis. However, data from epidemiological and genetic studies do not support the concept of linear cancer progression [38, 39]. The parallel progression model addresses these important issues—this concept places tumor cell dissemination and metastatic growth into the earliest stages of the disease, long before the tumor becomes clinically apparent. Accordingly, cells that enter the circulation and persist in secondary sites are still evolving, thus leading to development of divergent cell populations adapted to specific microenvironments. In our

study, removal of the primary tumor did not affect the phenotype of circulating tumor cells. Moreover, their expression profiles did not reflect the properties of the primary tumor. It may be, therefore, assumed that the detected CTC population left the primary tumor long before diagnosis and subsequently re-entered the circulation from a secondary homing site (e.g. bone marrow). These observations strongly support the parallel progression model. Alternatively, it is possible that only a small subpopulation of cancer cells is able to leave the primary tumor and enter blood circulation. These pre-selected cells do not reflect general properties of primary tumor and thus represent only a minor percentage of all cancer cells. Assuming that stem cell-like cancer cells are the active source of metastatic spread, it seems likely that such cells should be detectable among this particular subpopulation [22].

Conclusions

In the present report, we showed that the removal of the primary tumor has no impact on the phenotype of circulating tumor cells. In addition, the phenotype of CTC does not reflect that of the primary tumor. This suggests that CTC are not an epiphenomenon of primary tumors but instead have a biological significance of their own. That is, CTC may reflect a subpopulation of the primary tumor able to disseminate and migrate and are able to persist in secondary homing sites to subsequently re-enter the circulation. So far, studies on circulating tumor cell phenotype were limited by small sample sizes and methodological differences. A prospective analysis involving large numbers of patients is necessary to verify these hypotheses. The introduction of standardized diagnostic assays, such as AdnaTest and CellSearch, may facilitate such analysis. The implications for adjuvant therapy will require further discussion.

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Conflict of interest The authors declare that they have no conflicts of interests.

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Prognostic relevance of induced and spontaneous apoptosis of disseminated tumor cells in primary breast cancer patients

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Abstract

Background: An imbalance between cell proliferation and programmed cell death can result in tumor growth. Although most systemic cytotoxic agents induce apoptosis in tumor cells, a high apoptotic rate in primary breast cancer correlates with poor prognosis. The aim of this study was to investigate the incidence and the prognostic significance of apoptotic disseminated tumor cells (DTC) in the bone marrow (BM) of breast cancer patients who either underwent primary surgery or primary systemic chemotherapy (PST).

Methods: A total of 383 primary breast cancer patients with viable DTC in the BM were included into this study. Eighty-five patients were initially treated with primary systemic chemotherapy whereas 298 patients underwent surgery first. Detection of apoptotic DTC were performed by immunocytochemistry using the M30 antibody which detects a neo-epitope expressed after caspase cleavage of cytokeratin 18 during early apoptosis. The median follow up was 44 months (range 10–88 months).

Results: Eighty-two of 298 (27%) primary operated patients and 41 of 85 (48%) patients treated with primary systemic systemic therapy had additional apoptotic DTC (M30 positive). In the neoadjuvant group M30-positive patients were less likely to suffer relapse than those without apoptotic DTC (7% vs. 23% of the events, $p = 0.049$). In contrast, the detection of apoptotic DTC in patients treated by primary surgery was significantly associated with poor overall survival (5% vs. 12% of the events, $p = 0.008$).

Conclusions: Apoptotic DTC can be detected in breast cancer patients before and after systemic treatment. The presence of apoptotic DTC in patients with PST may be induced by the cytotoxic agents. Thus, both spontaneous and chemotherapy-induced apoptosis may have different prognostic significance.

Keywords: Apoptosis, M30, Breast cancer, Survival, Disseminated tumor cell

Background

30-40% of primary breast cancer patients present with disseminated tumor cells (DTC) at the time of diagnosis and the detection of these cells in the bone marrow has been shown to be a strong independent prognostic factor for disease free and overall survival [1]. Furthermore, DTC are able to survive systemic treatment and their persistence is associated with a poor outcome [2]. However, not all of these patients develop distant metastatic disease during follow-up suggesting that the majority of detected DTC has a short half-life and not the capability to induce tumor growth at secondary sites ("metastatic inefficiency"). Beyond mere detection of DTC, it is therefore important to further characterize these cells with respect to their phenotype and apoptotic status [3].

Apoptosis is a strongly regulated process that occurs in biological organisms leading to destruction of individual cells [4,5]. The role of this programmed cell death in oncogenesis has been intensively investigated in the past two decades. Since the survival of genetically altered cells results in carcinogenesis, an inadequate ratio of apoptosis

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leads to uncontrolled cell proliferation and thereby tumor growth. This is considered to be a result of mutations in oncogenes which are responsible for the regulation of apoptosis, including BCL-2, C-MYC and P53 [6-8]. Paradoxically, several studies have shown that a high ratio of apoptotic cells in untreated primary breast cancer generally correlates with increased cell proliferation, negative hormonal status, high grading and thus with a poor clinical outcome [8-10]. Whether this phenomenon is restricted to primary tumor or if it takes place in DTC remains to date unclear.

In contrast, chemotherapeutic agents can induce apoptosis of tumor cells leading to disease regression. This process can be explored *in vivo* in patients treated with PST [11]. We have previously reported that PST may induce apoptosis not only in primary tumor, but in DTC as well [3].

The purpose of this study was to investigate the incidence and prognostic significance of apoptotic DTC in two different subgroups of primary breast cancer patients: 1) patients who underwent surgery first and 2) patients treated with PST.

Methods

A total of 383 primary breast cancer patients treated between 2003 and 2009 at the Department of Obstetrics and Gynecology, University of Tuebingen, Germany were enrolled in this study, which was approved by the local research ethics committee (560/2012R). Inclusion criteria were: non metastatic breast cancer (T1-T3, N0-3, M0) and DTC positive BM status. Patients were subdivided into two groups based on their treatment schedule: (1) patients with surgery followed by adjuvant treatment (n = 298), and (2) patients with PST (n = 85). The clinical characteristics of patients are presented in Tables 1 and 2. The clinical response to PST was assessed by ultrasound, mammography and physical examination and was defined according to the World Health Organization criteria [12]. Pathological complete response was considered in patients with absence of invasive tumor in the breast and negative lymph node status. BM aspiration was performed intraoperatively in both groups.

Table 1 Clinical data for patients who underwent primary surgery

	n	M30 positive DTC	p-value
	N = 298	(%)	
Total	298	82 (27)	
Menopausal status			n.s.
Premenopausal	63	14 (22)	
Postmenopausal	235	68 (29)	
Tumor size			n.s.
pT1	204	57 (28)	
pT2-4	94	25 (27)	
Nodal status			n.s.
Negative	214	54 (25)	
Positive	84	28 (33)	
Histology			n.s.
Ductal	226	63 (28)	
Lobular	54	16 (30)	
Others	18	3 (17)	
Grading			n.s.
I/II	268	74 (28)	
III	30	8 (27)	
ER status			n.s.
Negative	45	12 (27)	
Positive	253	70 (28)	
PR status			n.s.
Negative	53	11 (21)	
Positive	245	71 (29)	
HER2 status			n.s.
Negative	232	69 (30)	
Positive	52	12 (23)	

Collection and analysis of BM

Between 10 and 20 ml of BM were aspirated from the anterior iliac crest and processed within 24 hours. All specimens were obtained after written informed consent from patients. BM samples were separated by density centrifugation over Ficoll (Biochrom, Germany) with a density of 1,077 g/ml. 10^6 mononuclear cells were spun onto a glass slide using a cytocentrifuge (Hettich, Tuttlingen, Germany). Slides were than fixed in a 4% neutral buffered formalin solution for 10 minutes and were rinsed in phosphate-buffered saline. Automatic immunostaining was performed on the DAKO autostainer using the monoclonal mouse A45–B/B3 antibody (Micromet, Munich, Germany), and the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturers' instructions. For each patient, 2×10^6 cells were analyzed. Slides were automatically scanned using the ACIS™ imaging system (ChromaVision, Medical Systems Inc., San Juan, Capistrano, CA, USA) and evaluated based on the recommendations for standardized tumor cell detection and the criteria of the European ISHAGE Working group [13,14]. The MCF-7 cell line served as a positive control. Leukocytes from healthy volunteers were used as a negative control. All BM specimens were evaluated qualitatively, as positive and negative for DTC.

383 primary breast cancer patients with A45-B/B3 positive DTC in BM were included into this study. In order to evaluate the apoptotic status of DTC in this

Table 2 Clinical data for patients who underwent neoadjuvant therapy

	n	M30 positive DTC	p-value
	N = 85	(%)	
Total	85	41 (48)	
Menopausal status			n.s.
Premenopausal	48	20 (42)	
Postmenopausal	37	21 (57)	
Tumor size			n.s.
ypT0	19	12 (63)	
ypT1	31	13 (42)	
ypT2-4	35	16 (46)	
Nodal status			0.007
ypN negative	39	25 (64)	
ypN positive	46	16 (35)	
Histology			n.s.
Ductal	66	32 (49)	
Lobular	15	7 (47)	
Others	4	2 (50)	
Grading			n.s.
I/II	67	34 (50)	
III	18	7 (39)	
ER status			n.s.
Negative	28	15 (54)	
Positive	57	26 (46)	
PR status			n.s.
Negative	27	17 (63)	
Positive	58	24 (41)	
HER2 status			n.s.
Negative	66	31 (47)	
Positive	19	10 (53)	

group, additional BM slides were stained using the M30 antibody (Roche Applied Science, Mannheim, Germany) and analyzed by use of the APAAP kit detection method as described above. The M30 antibody reacts with a neoepitope expressed only after caspase cleavage of cytokeratin 18 during early apoptosis [15]. M30 antibody does not bind intact, full-length cytokeratin 18 in viable or necrotic cells and can, therefore, be used specifically to recognize apoptotic cells [16]. Identification of apoptotic DTC was based on positive M30 staining and cytomorphological criteria as described elsewhere [17-19]. MCF-7 cells treated with sodium azide served as a positive control; untreated MCF-7 cell line and leukocytes from healthy volunteers were used as a negative control. Figures 1 and 2 show M30 and pan-cytokeratin staining.

Statistical analysis

The chi-squared test was used to evaluate the association between apoptotic DTC and clinicopathological factors. Statistical analysis was performed by SPSS (version 19). Values of $p < 0.05$ were considered statistically significant. Survival intervals were measured from the time of BM biopsy until death or the first diagnosis of relapse. Relapse was defined as either local recurrence or distant metastasis. Survival was calculated using Kaplan-Meier method and compared by the log-rank test.

Results

Patients characteristics

A total of 383 DTC positive breast cancer patients were included in this study. Two-hundred ninety-eight patients underwent breast surgery first. 204 out of 298 patients (68%) had T1 tumors and 214 (71%) were node negative. The most common histological tumor type was invasive ductal carcinoma. Estrogen and progesterone receptor status were positive in 85% and 82% of these patients, respectively. Fifty-two of 284 (18%) patients had HER2 positive tumors. Clinical data of this group are summarized in Table 1. Eighty-five patients were treated with PST. The majority of these patients was premenopausal (48 of 85 cases). 22% achieved pathological complete response after chemotherapy while 53% responded partially. Stable disease was observed in 19% of patients whereas 6% of patients (5 cases) developed progressive disease (Table 3). Clinical data of patients treated with PST are summarized in Table 2.

Presence of apoptotic DTC in patients treated with primary surgery

In eighty-two of 298 (27%) patients with pan-cytokeratin positive DTC in BM who underwent primary surgery additional apoptotic DTC could be detected. No correlation could be found between positive M30 status and any established prognostic factors, including tumor size, lymph node status, hormone receptor status or grading.

Presence of apoptotic DTC in patients treated with PST

Forty-one of 85 (48%) patients had additional M30 positive DTC after completion of PST. Patients with apoptotic DTC were less likely to have nodal metastasis (35% vs. 64%; p = 0.007). No significant correlation could be observed between the positive M30 status of DTC and other clinicopathological factors. The presence of apoptotic DTC was associated with response to PST. M30 positive cells were found in 63% of patients with complete remission, 53% with partial remission and 31% with stable disease, respectively. None of the patients with progressive disease had M30 positive DTC (p = 0.034; Table 3).

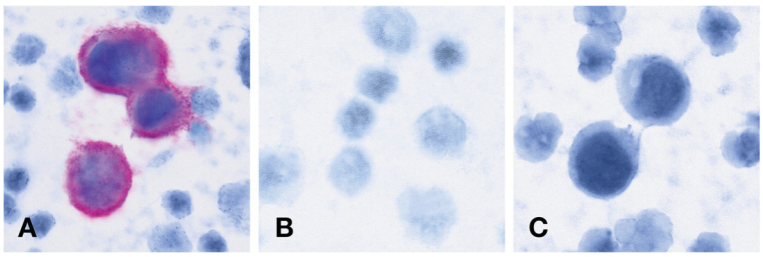

Figure 1 M30 control stainings (A) Cluster of M30 positive apoptotic MCF-7 cells with leukocytes in the background (positive control) (B) Leukocytes from healthy volunteers (negative control) (C) Cluster of M30 negative viable MCF-7 cells with leukocytes in the background (negative control).

Survival analysis

The median follow-up was 44 months (range: 10–88 months). 32 of 383 patients were diagnosed with relapse (either local recurrence or distant metastasis) and 28 died during follow-up. Clinical outcome data are summarized in Table 4.

Survival analysis of neoadjuvant patients

Thirteen of 85 neoadjuvant patients (15%) presented with relapse during follow-up. Patients with additional M30 positive DTC were less likely to suffer from relapse than patients with only non-apoptotic DTC (7% vs. 23%; $p = 0.049$). However, the association between disease-free interval and M30 status of DTC assessed by Kaplan-Meier analysis did not reach statistical significance ($p = 0.128$; 70 months, 95% CI: 63–78 months vs. 81 months, 95% CI: 74–87 months). Seven of 85 neoadjuvant patients (8%) died during follow-up. No correlation could be found between M30 status of DTC and overall survival in this group of patients.

Survival analysis of patients treated with primary surgery

Twenty-one out of 298 (7%) patients in this group died during follow-up. The overall survival was significantly shorter among patients with M30 positive DTC as compared to M30 negative patients (75 months, 95% CI: 68–81 months vs. 84 months, 95% CI: 81–86 months; $p = 0.008$). However, there was no association between disease free survival and M30 status (83 months in M30 positive patients, 95% CI: 80–85 months vs. 78 months in M30 negative patients, 95% CI: 73–84 months; $p > 0.05$). Figure 3 shows the Kaplan-Meier analysis of overall survival in patients who underwent primary surgery.

Discussion

The presence of DTC in BM of patients with primary breast cancer is an independent prognostic factor associated with poor clinical outcome [1]. Although this phenomenon can be seen in 30-40% of breast cancer patients, only a minority of DTC positive patients will develop distant metastasis in course of disease ("metastatic inefficiency").

The aim of the present study was to evaluate the incidence and prognostic relevance of apoptotic DTC in breast cancer patients. We focused on two subsets of patients: 1) untreated patients whose bone marrow status was assessed at the time of primary surgery, and 2) pretreated patients after completion of neoadjuvant cytotoxic therapy.

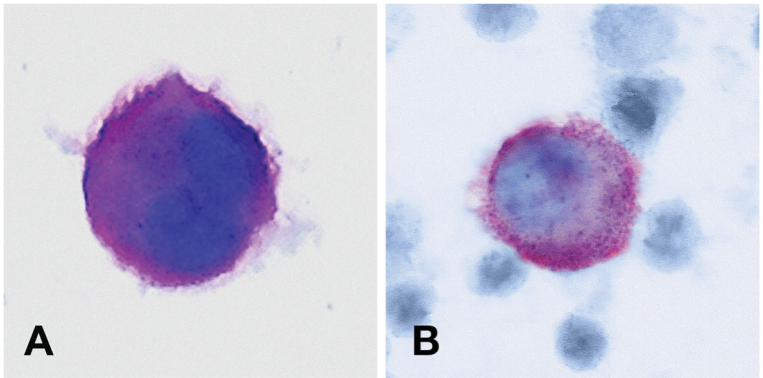

Figure 2 Pan-cytokeratin and M30 staining of DTC from primary breast cancer patients. (A) A45–B/B3 positive viable DTC from a primary breast cancer patient **(B)** M30 positive apoptotic DTC from a primary breast cancer patient. The typical morphology of a tumor cell can be recognized (positive cytokeratin-staining, large nucleus, high nuclear to cytoplasmic ratio, nucleus partially covered by cytokeratin staining, nucleus granular).

Table 3 Apoptotic DTC and clinical response to neoadjuvant chemotherapy

	N (%)	M30 positive DTC	%
Total	85 (100)	41	48
Complete remission	19 (22)	12	63
Partial remission	45 (53)	24	53
Stable disease	16 (19)	5	31
Progressive disease	5 (6)	0	0

Clinical relevance of apoptotic DTC in untreated patients at time of primary surgery

M30 antigen is an early apoptotic marker of epithelial cells, detectable after caspase cleavage of cytokeratin 18. This antibody was used in our study to assess the apoptotic status of DTC in 298 patients who received no treatment prior to surgery. Patients with apoptotic DTC had a significantly shorter overall survival compared to patients with only non-apoptotic DTC (75 months vs. 84 months, $p < 0.008$). This seems not conclusive, since apoptotic DTC are generally assumed to result from a phenomenon described as "metastatic inefficiency". According to this conceptual model, only a small percentage of tumor cells are able to survive and persist at secondary homing sites. Large numbers of cancer cells are shed from the primary site into the systemic circulation; only a small subset will give rise to overt metastases. To successfully reach and colonize a secondary site, a tumor cell must complete a series of steps (metastatic cascade): migration from the primary tumor, intravasation into the blood stream, survival of the vigorous passage in blood, extravasation, and development of micrometastases in distant organs [20]. Failure in any one of these steps leads to elimination of tumor cells; 99,9% of shed cells are thought to perish during the process, while only a minor subpopulation attains metastatic capacity [21]. The key regulatory points that contribute to metastatic inefficiency remain unclear; initiation of apoptosis has been assumed to be a major component of this mechanism. Yet, in our study apoptotic status of DTC in bone marrow in untreated patients resulted in significantly worse survival.

Table 4 Survival analysis of patients depending on M30 status of DTC

	Patients with primary surgery	Patients with NST
N	298	85
Deaths	21	7
M30 positive	10/82 (12%)	4/41 (10%)
M30 negative	11/216 (5%)	3/44 (7%)
P	0.032	n.s.
Overall survival*		
(M30 positive vs M30 negative)	0.008	n.s.
Relapses†	19	13
M30 positive	7/82 (9%)	3/41 (7%)
M30 negative	12/216 (6%)	10/44 (23%)
P	n.s.	0.049
Relapse free survival*	n.s.	0.128
(M30 positive vs M30 negative)		

*Calculated by log-rank test. † Including local recurrence and distant metastases.

In the past decades, major research efforts have been conducted to study the role of apoptosis in the primary tumor [10,22]. The association between apoptosis rates and cell proliferation is well established; increased apoptosis reflects a high cell turnover in the tumor. In breast cancer, high levels of apoptosis correlate with enhanced cellular proliferation and biological markers of increased malignancy, such as negative hormone receptor status, high histological grade, HER2 overexpression, positive lymph nodes, tumor aneuploidy and a decreased expression of bcl-2 protein [10,23,24]. Furthermore, high apoptotic counts are associated with shortened disease-free and overall survival [9,10,24]. Similar observations were made in other solid tumors, including prostate and bladder cancer [25,26]. These data seem to disprove the concept that the elimination of apoptosis in tumor cells is a necessary condition for autonomous uncontrolled cancer growth. On the contrary, an enhanced rate of spontaneous apoptosis in the primary tumor is an indicator of high proliferation and negative prognostic markers.

Clinical relevance of therapy-induced apoptosis in DTC

Over the last two decades, neoadjuvant systemic therapy (NST) has become the standard treatment strategy for locally advanced breast cancer, conducted primarily to enhance the possibility of breast-conserving surgery [27]. Beyond this indication NST offers the additional possibility to test in vivo the chemosensitivity of the primary tumor [28]. The pathological response to NST is associated with favorable clinical outcome and is considered by some as a surrogate marker for complete eradication of micrometastatic disease. However, up to 25% of patients who achieve complete pathological remission will suffer relapse within five years of diagnosis, suggesting subclinical persistence of isolated tumor cells beyond systemic treatment.

We reported previously a high incidence of persistent DTC after NST [3]. Since apoptosis is the main mechanism of chemotherapy-induced disease regression [29], we aimed to assess the apoptotic status of DTC in patients who received neoadjuvant therapy. In 41 out of 85 (48%) DTC positive patients additional apoptotic tumor cells were detected after therapy. Compared to patients with only viable DTC, patients with apoptotic DTC responded better to therapy, reaching complete or partial remission in 88% (vs. 64% in the M30-negative group, $p = 0.034$).

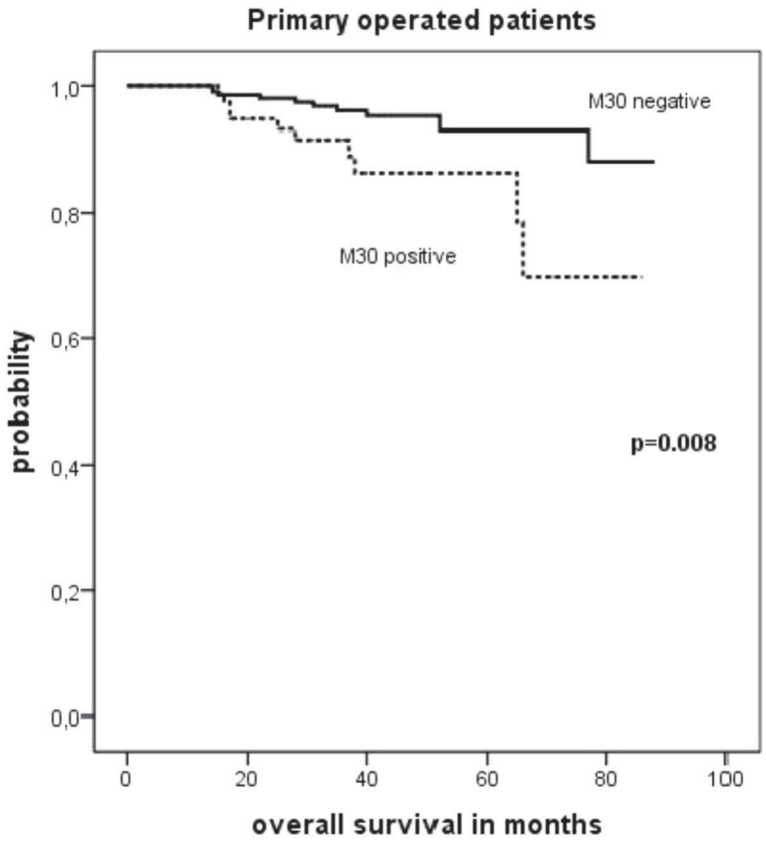

Figure 3 Kaplan-Meier survival analysis depending on M30 status of DTC in bone marrow of previously untreated patients (overall survival).

Moreover, additional apoptotic cells in BM were significantly associated with negative nodal status after completion of neoadjuvant treatment but not with any other clinicopathological factor. Pathological response of the primary tumor and lymph nodes metastases was therefore reflected by changes in tumor cells in secondary sites, such as bone marrow. Further, patients with positive M30-status were less likely to suffer from a relapse ($p = 0.049$). This is in accordance with clinical studies: patients who achieve complete pathological response to neoadjuvant treatment regime perform favorably with regard to overall and disease-free survival [30]. In addition, patients converted to node negative disease after neoadjuvant treatment have high survival rates, despite residual tumor in the breast [31]. These data indicate that in these patients apoptotic tumor cells are associated with therapy response.

Conclusions

Although the observations made in primary tumors cannot be directly extrapolated to DTC in secondary sites, our data suggest that high level of spontaneous apoptosis in minimal residual disease (MRD) is an indicator of poor prognosis. Hypothetically, if the presence of apoptotic DTC reflects an active status of MRD; accordingly, dormant (inactive) tumor cells would appear as non-apoptotic. In contrast, therapy-induced apoptosis of DTC was correlated to pathological response of the tumor and may be regarded as a favorable event. Our data demonstrate for the first time that the biological significance of apoptotic status of DTC is contingent on whether the apoptosis occurs spontaneously or was induced by treatment.

Abbreviations

ACIS: Automated cellular imaging system; APAAP: Alkaline phosphatase-antialkaline phosphatase; BM: Bone marrow; CK: Cytokeratin; CTC: Circulating tumor cell; DTC: Disseminated tumor cell; HE: Hematoxylin-eosin; MRD: Minimal residual disease; NST: Neoadjuvant systemic therapy; n.s.: Not significant.

Competing interests

The authors declare that they have no competing interest.

Authors' contribution

NK designed the study, performed the statistical analysis and drafted the manuscript. AH made substantial contributions to interpretation of data, performed the survival analysis and helped to draft the manuscript. MB and FMS made substantial contributions to interpretation of data and helped to draft the manuscript. AS made substantial contributions to interpretation of data. MW, CR, JH and MH participated in data collection and study coordination. TF designed the study and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Determination of the androgen receptor status of circulating tumour cells in metastatic breast cancer patients

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Abstract

Background: The prognostic relevance of circulating tumour cells (CTCs) in metastatic breast cancer (MBC) patients has been confirmed by several clinical trials. However, predictive blood-based biomarkers for stratification of patients for targeted therapy are still lacking. The DETECT studies explore the utility of CTC phenotype for treatment decisions in patients with HER2 negative MBC. Associated with this concept is a plethora of translational projects aiming to identify potential predictive biomarkers. The androgen receptor (AR) is expressed in over 70% of hormone receptor-positive and up-to 45% of triple-negative tumours. Studies has indicated the promising nature of AR as a new therapy target with a clinical benefit rate for anti-AR treatment in MBC patients up to 25% The aim of this analysis was the characterization of CTCs regarding the expression of the AR using immunofluorescence.

Methods: MBC patients were screened for the HER2-status of CTCs in the DETECT studies. In a subset of CTC-positive patients ($n = 67$) an additional blood sample was used for immunomagnetic enrichment of CTCs using the CellSearch® Profile Kit prior to transfer of the cells onto cytospin slides. Establishment of immunofluorescence staining for the AR was performed using prostate cancer cell lines LNCaP and DU145 as positive and negative control, respectively. Staining of DAPI, pan-cytokeratin (CK) and CD45 was applied to identify nucleated epithelial cells as CTCs and to exclude leucocytes.

Results: Co-staining of the AR, CK and CD45 according to the above mentioned workflow has been successfully established using cell lines with known AR expression spiked into the blood samples from healthy donors. For this translational project, samples were analysed from 67 patients participating in the DETECT studies. At least one CTC was detected in 37 out of 67 patients (56%). In 16 of these 37 patients (43%) AR-positive CTCs were detected. In eight out of 25 patients (32%) with more than one CTC, AR-positive and AR-negative CTCs were observed.

Conclusion: In 43% of the analysed CTC samples from patients with MBC the AR expression has been detected. The predictive value of AR expression in CTCs remains to be evaluated in further trials.

Keywords: Predictive marker, Androgen receptor, Metastatic breast cancer, Circulating tumour cells

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Background

Breast cancer (BC) is the most common malignancy in women, with almost 1.7 million new cases diagnosed per year [1]. While localized disease has become increasingly treatable, with an average 5-year survival rate of approximately 90%, metastatic breast cancer (MBC) still carries a very poor prognosis. Despite a complete removal of the tumour and adequate systemic treatment, 25–30% of primary BC patients suffer from a distant recurrence during the follow-up, making metastatic BC the second leading cause of cancer-related death among women worldwide [1–3]. Therefore, novel therapeutic targets and innovative systemic treatment approaches in MBC are still desperately required. The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor family, thus sharing several features with the oestrogen (ER) and progesterone receptors. In its unbound state, the AR is located in the cytoplasm in complex with heat shock protein 90 and other chaperone proteins. Upon ligand stimulation, the AR undergoes dimerization and translocates to the nucleus, where it regulates transcription by binding to target genes [4–6]. AR *expression has been reported* in over 70% of all primary BCs and it is more often detected in ER-positive than in ER-negative tumours. However, up to 45% of triple negative BC patients express the AR [7–14]. The role of the AR in BC has not yet been completely elucidated and seems to depend on tumour subtype. Several in vitro studies have shown a divergent effect of androgens on cell proliferation in BC cell lines [15, 16]. In the presence of ERα, the AR can either have proliferative or anti-proliferative activity, depending on the level of the co-expressed ERα and the availability of the respective ligand [17–19], Moreover, an AR-overexpression in HR-positive BC has been shown to be associated with resistance to tamoxifen, which may be reversed by an anti-androgen treatment [20]. In contrast, in HER2-positive and triple negative BC a proliferative function of the AR seems to be consistent [21]. The above indicates a strong rationale to explore AR expression as a therapeutic target in all subtypes of BC. Anti-AR treatment has recently been evaluated in two multicentre phase II studies on MBC patients showing promising results with a clinical benefit rate of up to 25% [22, 23]. The ongoing trials on anti-androgen treatment in breast cancer are summarized in Table 1. However, none of these trials included the AR-status of CTCs for stratification. Circulating tumour cells (CTCs) can be detected in approximately 40–80% of MBC patients and predict impaired clinical outcome

Table 1 Ongoing trials on anti-androgen treatment in breast cancer

Study	Status	Estimated Enrollment	Condition	Intervention	Primary Endpoint
NCT00468715 (Phase II) non-randomized	Active, not recruiting	28	AR+/HR- MBC	• Bicalutamide	CBR[a] (observed CBR of 19% [22])
NCT01889238 (Phase II) non-randomized	Active, not recruiting	118	AR+/ triple negative ABC	• Enzalutamide	CBR (observed CBR of 25% [24])
ENDEAR trial NCT02929576 (Phase III)	withdrawn	780	Triple negative ABC	• Enzalutamide vs • Paclitaxel vs • combination	PFS
NCT02750358 (phase II) non-randomized, single agent	Active, not recruiting	200	AR+ / triple negative ESBC	• Enzalutamide	treatment discontinuation rate/ feasibility
NCT02689427 (phase IIb) non-randomized	recruiting	37	AR+ / triple negative ESBC	• Enzalutamide plus Paclitaxel in neoadjuvant setting	PCR rate
NCT02007512 (phase II) randomized	Active, not recruiting	247	HR+ HER2- ABC	• Exemestan +/− Enzalutamide	PFS
NCT02463032 (Phase II) randomized	Active, not recruiting	88	ER+/AR+ ABC	• GTx-024 (Enobosarm) • SARM • 9 vs. 18 mg.	CBR
NCT01990209 (phase II) non-randomized	Active, not recruiting	86	HR+/AR+ or triple negative /AR+ MBC	• TAK-700 (orteronel) a nonsteroidal inhibitor of CYP17A1	RR[b] DCR[c]
NCT02067741 SAKK21/12 (Phase II) non- randomized	active, not recruiting	90	HR+/HER2- or triple negative/ AR+ ABC	• transdermal CR1447 (4-OH-testosterone)	DCR
NCT02091960 (Phase II) non-randomized	Active, not recruiting	103	HER2 + /AR + ABC	• Enzalutamide + trastuzumab	CBR

AR androgen receptor, *ER* oestrogen receptor, *PR* progesteron receptor, *HR* hormone receptor, *HER2* human epidermal growth factor receptor 2:, *CBR* Clinical benefit rate, [a] defined as proportion of patients with stability, partial response and complete response assessed by RECIST v1.1 criteria, *PFS* progression free survival, *ESBC* early stage breast cancer, *SARM* selective androgen receptor modulator, *ABC* advanced breast cancer (metastatic or locally advanced), *RR* responder rate, [b] defined as the percentage of complete and partial responders (CR + PR) assessed by RECIST v1.1 criteria, *DCR* disease control rate, [c] defined as the percentage of patients who do not exhibit progression

[25]. Beyond their prognostic significance, CTCs may serve as a "liquid biopsy", since their expression profile is assumed to most adequately reflect the phenotype of the presently dominant tumour cell population in metastatic disease. Moreover, a CTC phenotype may potentially predict the response to treatment, thereby making these cells not only a valuable source of cancer material but also a potential target for a therapeutic intervention [26]. The clinical utility of CTCs in driving treatment decisions is currently being evaluated within the DETECT studies [27]. The aim of the present substudy was to evaluate the AR status of CTCs in a cohort of MBC.

Methods

Patient material

Blood samples from 67 MBC patients, screened within the German DETECT III/IV trials (III: NCT01619111, IV: NCT02035813) between 2012 and 2017 for the HER2-status of CTCs, were eligible for this analysis (for more information: www.detect-studien.de). DETECT III/IV study trial is a multicenter study program for patients with HER2-negative MBC and circulating tumor cells. The main objective of this study is to evaluate the efficacy of personalized breast cancer therapy based on the presence and phenotype of CTCs. The flow chart of our substudy is presented in Fig. 1. Written informed consent was obtained from all participating patients and the study was approved by the Ethical Committee of the Eberhard Karls University of Tuebingen (responsible for DETECT III: 525/2011AMG1) and the local Ethical Committee of the Heinrich Heine University of Duesseldorf (DETECT III: MC-531; DETECT IV: MC-LKP-668).

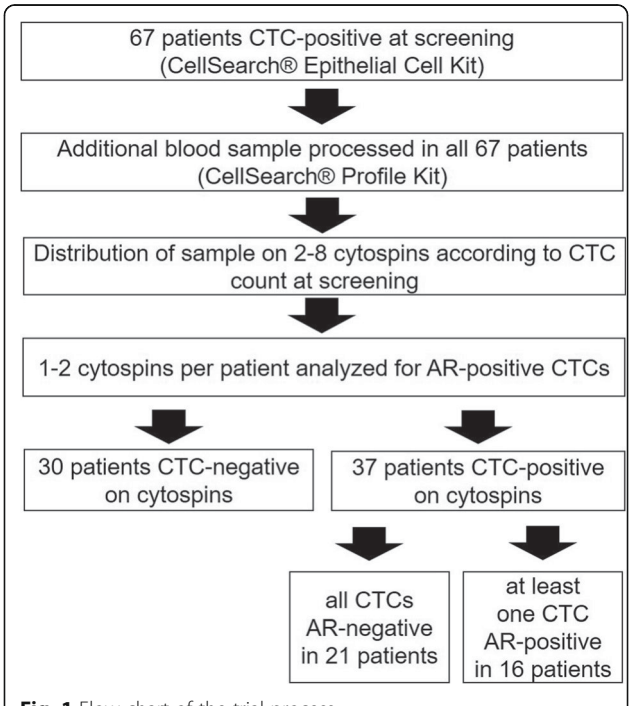

Fig. 1 Flow chart of the trial process

CTC enrichment and cytospin preparation

Blood samples were drawn into 10 ml CellSave tubes (Menarini Silicon Biosystems), maintained at room temperature and processed within 72 h after collection. The CellSearch® Epithelial Cell Kit (Menarini Silicon Biosystems) was used routinely for enrichment and enumeration of CTCs as described previously [28]. In a subset of CTC-positive patients an additional blood sample was processed using the CellSearch® Profile Kit (Menarini Silicon Biosystems) to enrich tumour cells expressing the epithelial cell adhesion molecule (EpCAM) immunomagnetically without further labelling or enumerating the cells. 10 mL of blood from the CellSave Preservative Tube was transferred into a correspondingly labelled 15 mL CELLSEARCH® Conical Centrifuge Tube with 6.5 mL of dilution buffer, consisting of phosphate buffered saline (PBS), 0.5% bovine serum albumin and 0.1% sodium azide. The sample was centrifuged at 800 x g for 10 min at room temperature and processed on the CELLTRACKS® AUTOPREP® System within 1 h. The magnetic incubation steps were performed and the vast majority of leukocytes and other blood components were depleted from the final sample. Using a ROTOFIX 32 A centrifuge (800 rpm, 2 min; Hettich GmbH & Co.KG, Tuttlingen, Germany) 400 μl of the white blood cell-depleted cell suspension were spun onto a glass slide. The slides were air-dried overnight at room temperature and stored at − 20 °C. One to two cytospins per patient was analysed for AR-positive CTCs. Control cytospins with AR-positive LNCaP cells and AR-negative Du145 cells mixed with peripheral blood mononuclear cells (PBMCs) from a healthy volunteer were similarly prepared, stored and fixed.

Androgen receptor staining

Cytospins were thawed at room temperature in a humid chamber for approximately 20 min and fixed with CellSave (Veridex, Warren, NJ, USA) for 10 min. After an initial wash step with PBS (Sigma, Munich, Germany), cells were permeabilized with PBS containing 0.1% Triton X-100 for a period of 10 min prior to blocking with Protein Block solution (DAKO, CA, USA) for another 10 min. The immunofluorescence stainings were performed using the Androgen Receptor (D6F11) XP rabbit monoclonal antibody (1:100, Cell Signaling Technologies Inc., Cambridge UK) and the pan-cytokeratin (CK) antibody (C11) directly conjugated to fluorescein isothiocyanate (FITC) (1:100, Sigma, Munich, Germany) for 60 min. Cytospins were subsequently incubated with a secondary donkey anti-rabbit antibody, labelled with Alexa Fluor 594 (1:500, Invitrogen Molecular Probes, Carlsbad, CA, USA) and an Alexa Fluor 647-conjugated CD45

antibody (35-Z6) (1:20, Santa Cruz Biotechnology, Dallas, TX, USA) for 30 min. Nuclear DNA staining was performed with 4′6-diamidino-2-phenylindole (DAPI) in mounting media (Vector Laboratories, Burlingame, CA, USA). Preparations of the prostate cancer cell line LNCaP mixed with PBMCs from a healthy volunteer served as a positive control for CK and AR staining. The AR-negative control slides of Du145/PBMC mixtures were also included with each batch of samples. CK positive, CD45 negative cells that contained an intact nucleus (DAPI positive) were identified as CTCs. Positive and negative control stainings are shown in Fig. 2.

Statistical analysis

The chi-squared test was used to evaluate the association between CTCs and clinicopathological factors. Statistical analysis was performed by SPSS (version 25). Values of $p < 0.05$ were considered statistically significant.

Results

Patients` characteristics

Peripheral blood from 67 MBC patients screened for participation in the DETECT trial were eligible for this study. 55 patients (82%) had hormone receptor (HR)-positive/HER2-negative tumours, two cases (3%) had immunohistochemistry stainings indicating HR-positive/HER2-positive disease, and 10 patients (15%) had a triple negative breast cancer (TNBC). In 26 patients (40%) the blood draw was performed prior to the first line therapy for metastatic disease. The remaining 41 patients (60%) had progressive metastatic disease at blood sampling. The clinical data of the patients are summarized in Table 2.

CTC detection and AR expression in CTCs

At least one CTC was detected in 37 patients (56%). The CTC count ranged from 1 to 101 cells. In 16 out of the 37 CTC-positive patients (43%), AR-positive CTCs could be detected. The percentage of AR-positive CTCs among

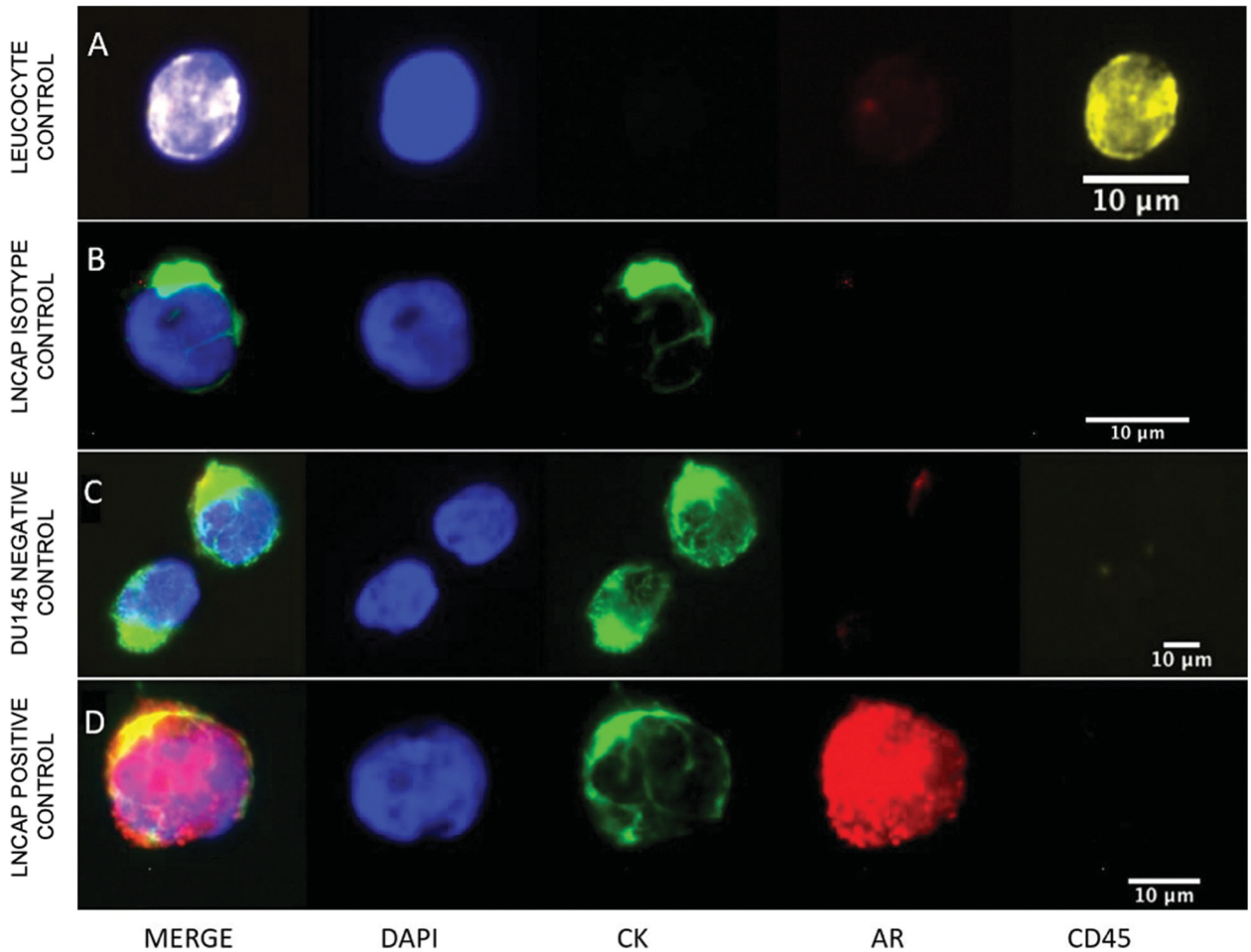

Fig. 2 Androgen receptor (AR) control stainings (**a**) CD45 positive control staining (leucocyte) (**b**) AR isotype control staining (LNCaP) (**c**) Du145 prostate cancer cell line (negative control) (**d**) LNCaP prostate cancer cell line (positive control)

Table 2 Clinical data of patients

	n N = 67	CTC positive (%)	*p*-value	AR-positive CTC (%)	p-value
Total	67	37 (55)		16 (43)	
Menopausal status			0.40		0.68
premenopausal	12	7 (58)		4 (57)	
postmenopausal	53	28 (53)		11 (39)	
unknown	2	2 (100)		1 (50)	
Line of treatment			0.75		0.30
1st	26	15 (58)		8 (53)	
≥ 2nd	41	22 (54)		8 (36)	
IHC tumour type			0.94		0.56
TNBC	10	6 (60)		2 (33)	
HR+/HER2-	55	30 (54)		14 (47)	
HR+/HER2 + [a]	2	1 (50)		0	
Site of metastasis			0.65		0.44
bone only	14	8 (57)		4 (50)	
other site	52	28 (54)		11 (39)	
unknown	1	1 (100)		1 (100)	

[a]screening failure

CTCs detected per patient ranged from 0 to 100% (mean 35.5, 95%-CI: 21.4–49.6%). In 5 out of 16 patients (31%) with AR-positive CTCs, the AR was localized in the nucleus whereas in 10 patients (62.5%) the AR signal was detected in the cytoplasm. Both nuclear and cytoplasmic localization were observed in only one patient (6.5%). Heterogenic AR localization in CTCs is depicted in Fig. 3. Among the 25 patients with more than one CTC, 14 had only AR-negative CTCs, and 3 had only AR-positive CTCs. In the remaining 8 patients (32%), AR-positive and AR-negative CTCs could be detected and the AR-positivity rate ranged from 12 to 83%. The characteristics of CTC-positive patients are demonstrated in Table 3.

Discussion

There is growing evidence on the potential role of androgens and the AR in the pathogenesis of breast cancer. The majority of ER-positive breast cancers and up to 45% of TNBC express the AR in tumour tissue, making this biomarker an interesting therapeutic target [7–14]. AR targeting drugs, like bicalutamide or enzalutamide, are currently being evaluated in clinical trials focussing on AR-positive MBC, with favourable clinical benefit

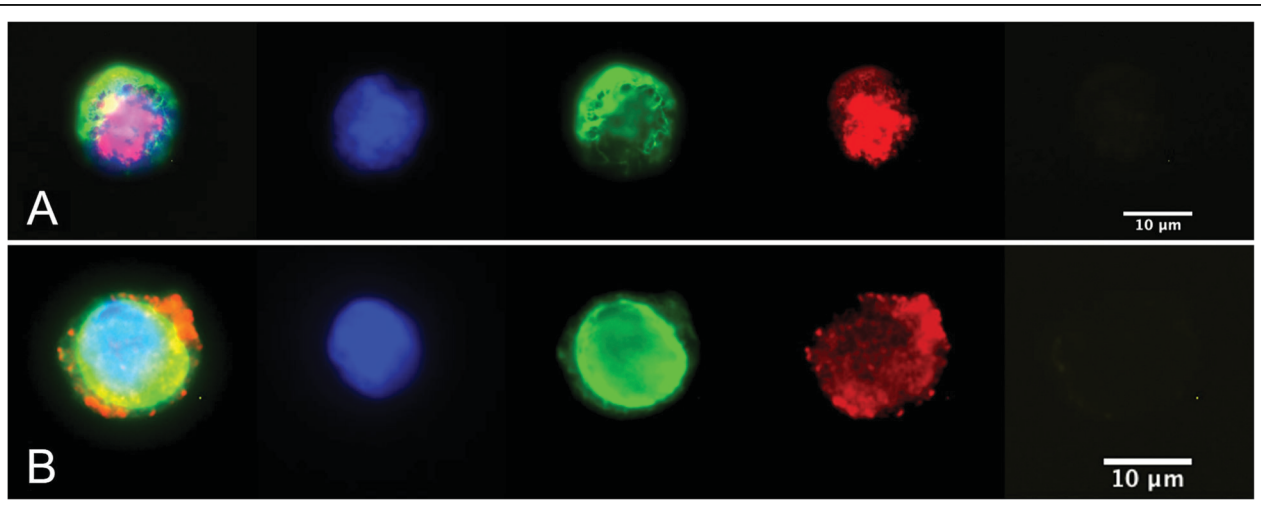

Fig. 3 androgen receptor (AR) staining of CTCs in metastatic breast cancer patients (**a**) AR-positive nuclear staining (**b**) AR-positive cytoplasmic staining

Table 3 Characteristics of CTC-positive patients

Patient	Menopausal status	IHC tumour type	Number of previously received treatment lines[a]	Metastatic site	CTC count	AR positive CTC (%)	AR localization
1	postmenopausal	HR+ HER2-	1	bone visceral	101	84 (83)	cytoplasm/ nucleus
2	premenopausal	HR+ HER2-	0	bone	13	7 (54)	cytoplasm
3	postmenopausal	HR+ HER2-	2	bone visceral	10	3 (30)	cytoplasm
4	postmenopausal	HR+ HER2-	2	bone	9	0 (0)	–
5	premenopausal	TNBC	0	bone visceral	8	1 (12)	cytoplasm
6	premenopausal	HR+ HER2-	0	bone	7	7 (100)	nucleus
7	unknown	HR+ HER2-	0	unknown	4	3 (75)	cytoplasm
8	postmenopausal	HR+ HER2-	4	bone visceral	4	3 (75)	cytoplasm
9	postmenopausal	HR+ HER2-	0	bone	3	1 (33)	cytoplasm
10	postmenopausal	HR+ HER2-	7	bone	3	3 (100)	cytoplasm
11	postmenopausal	HR+ HER2-	0	visceral	3	3 (100)	cytoplasm
12	postmenopausal	HR+ HER2-	3	bone visceral	3	0 (0)	–
13	postmenopausal	HR+ HER2-	4	bone visceral	3	0 (0)	–
14	postmenopausal	HR+ HER2-	1	bone visceral	3	0 (0)	–
15	unknown	HR+ HER2+	2	visceral	3	0 (0)	–
16	premenopausal	HR+ HER2-	0	bone lymph nodes	3	0 (0)	–
17	premenopausal	TNBC	1	bone visceral	2	1 (50)	nucleus
18	postmenopausal	HR+ HER2-	1	bone	2	0	–
19	postmenopausal	HR+ HER2-	2	bone visceral	2	0	–
20	postmenopausal	HR+ HER2-	2	bone visceral	2	0	–
21	postmenopausal	TNBC	0	visceral	2	0	–
22	postmenopausal	HR+ HER2-	2	bone lymph nodes	2	0	–
23	postmenopausal	HR+ HER2-	2	bone	2	0	–
24	postmenopausal	HR+ HER2-	1	bone visceral	2	0	–
25	premenopausal	HR+ HER2-	0	bone	2	0	–
26	postmenopausal	HR+ HER2-	1	bone visceral	1	1 (100)-	nucleus
27	postmenopausal	HR+ HER2-	0	bone visceral	1	1 (100)	nucleus
28	postmenopausal	HR+ HER2-	3	bone visceral	1	1 (100)	cytoplasm
29	postmenopausal	HR+ HER2-	0	Lymph nodes	1	1 (100)	nucleus
30	postmenopausal	HR+ HER2-	7	Bone lymph nodes	1	1 (100)	cytoplasm
31	postmenopausal	HR+ HER2-	0	visceral	1	0	–
32	postmenopausal	HR+ HER2-	0	bone visceral	1	0	–
33	premenopausal	TNBC	1	visceral	1	0	–
34	postmenopausal	HR+ HER2-	0	visceral	1	0	–
35	postmenopausal	HR+ HER2-	0	visceral	1	0	–
36	postmenopausal	TNBC	1	bone visceral	1	0	–
37	postmenopausal	TNBC	2	visceral	1	0	–

[a]for metastatic disease

rates of up to 25% being obtained [22, 24]. However, since AR expression is not routinely assessed on BC tissue, AR expression status of MBC is mostly unknown. Archived primary tumour tissue or a direct biopsy of the metastatic lesion is required to assess the AR expression status in cases where an AR-targeted therapy is considered [22, 24]. In light of this, CTCs might serve as a 'liquid biopsy' and an attractive non-invasive alternative to the biopsy of a metastasis [29]. We established a triple immunofluorescence staining for the AR in CTCs and show that AR-positive CTCs can be detected in the peripheral blood of MBC patients. These findings are concordant with recently published data by Fujii et al. [30]. We used the EpCAM-based CellSearch® Profile kit for CTC detection to facilitate the identification of only tumour cells of epithelial origin. CTCs were further identified by direct visualisation of CK-positive, CD45-negative cells that contained an intact nucleus (DAPI positive). In our study, 16 out of 37 CTC-positive MBC patients (43%) also yielded AR-positive tumour cells in the peripheral blood. This positivity rate is higher than in the study by Fujii et al., where 23% AR-positive CTCs were detected in CTC-positive MBC patients [30]. This discrepancy may be due to differences in patient characteristics. The majority of patients included in our trial had HR-positive disease (57/67 patients (85%) compared to only 43/68 patients (63%) in the Fujii et al. study) and this subtype has been previously reported to be more likely to express AR [7, 14, 30]. The AR positivity rate of CTCs in our small MBC cohort amounted 43%. However, this positivity rate is lower than that reported for primary breast cancer tissue [7–14], which raises the question whether the AR status of CTCs coincides with that of the primary tumour. In the study by Fujii et al., three out of seven patients (43%) demonstrated AR-positive CTCs despite AR-negative primary tumours [30]. Phenotypic differences between the primary tumour, metastatic lesions and CTCs, with regard to other predictive factors such as ER or HER2, are a known phenomenon [28, 31–34]. Rocca et al. reported an overall concordance rate of 65% for AR expression between primary tumours and metastases [35]. Due to the lack of available tumour tissue (most of the patients were initially treated outside our centre), no comparison of the AR status between the CTCs and the corresponding tumour or metastatic lesion could be performed in our patients collective. However, as CTCs are an accepted non-invasive liquid biopsy [29], we hypothesize that the detection of AR-positive CTCs in MBC patients could be useful as a predictive factor for anti-AR treatment. The efficacy of targeting the AR in MBC patients with AR-positive CTCs need to be evaluated in further studies. Contrary to previously published analyses, we observed a heterogeneous localization of ARs in CTCs, with five out of 16 patients showing only nuclear AR staining and the majority (10 out of 16) only cytoplasmic staining. Both, nuclear and cytoplasmic staining was observed in CTCs from one patient. Previous studies defined AR positivity in the tumour tissue as a nuclear staining with a cut off value of ≥1% or ≥ 10% positive tumour cells regardless of intensity [11, 22, 36, 37]. In the analysis of the ARs in CTCs in BC patients, Fujii et al. also only counted nuclear localization of the receptor as positive [30]. However, heterogeneous subcellular localization of AR is a known phenomenon [5, 6]. Reyes et al. reported a common cytoplasmic AR localization in CTCs in metastatic castration-resistant prostate cancer patients [38]. The nuclear or cytoplasmic localization of the AR may reflect receptor activity, which mainly depends on the absence or presence of the ligand and was demonstrated to vary between cell lines [39–41]. Androgen serum levels in women are generally much lower than in men [42, 43], possibly leading to the reduced activity of the AR in breast cancer patients, which may explain the cytoplasmic localization of the receptor in some cases. On the other side, a postmenopausal status or an endocrine therapy with aromatase inhibitors increase serum levels of androgens in BC patients, which could result in AR activation and nuclear translocation [44, 45]. Interestingly, only three out of five patients presenting CTCs with exclusively nuclear AR localization were postmenopausal, compared to nine out of ten patients with a solely cytoplasmic localization. Of note is the fact that none of these five cases received an aromatase inhibitor administration at the time of blood draw. The one patient presenting with both cytoplasmic and nuclear AR localization was a postmenopausal woman treated with letrozole at the time of sample collection. Another explanation of our findings could be the genetic aberration of the AR resulting in an impaired function of the receptor [46]. Specific mutations of the AR gene can diminish or abolish its nuclear translocation abilities despite ligand binding. Mutations can also cause constitutively active, nuclear-localised AR even in the absence of the ligand [47]. Another possible reason for cytoplasmic AR localization has been proposed by Koryakina et al. [48]. In their trial on the cell cycle dependent regulation of AR in prostate cancer cell lines, a cytoplasmic localization of the receptor was shown to be characteristic of mitotic cells [48]. This might explain the relatively high rate of cytoplasmatic localized AR in our study as mitotic CTCs seem to be a common event in advanced breast cancer [49]. Whether cytoplasmic ARs can be targeted by anti-AR drugs remains to be clarified [38]. In the recent study by Kumar et al., the AR nuclear staining in BC was shown to have the highest accuracy in predicting the anti-androgen therapy response, however, with a rather modest positive predictive value of 30% [50]. In consideration of the above it is clear that the clinical relevance of heterogeneous subcellular AR localization in CTCs requires additional evaluative trials.

Conclusion

The phenotypic characterization of CTCs, which might serve as a real-time liquid biopsy, is gaining in importance. This necessitates the identification of new predictive markers for systemic treatment in patients with MBC. The AR represents such a potential therapy target, since it is being expressed in all BC subtypes. In the present analysis we established a triple fluorescent staining of the AR in CTCs. The established robust method allowed for the direct visualization of the tumour cell and showed that AR-positive CTCs can be detected in MBC patients. AR localization in CTCs can vary and may be detected both in the nucleus and cytoplasm. Whether AR-positive CTCs are suitable to serve as a therapeutic biomarker and whether the pleiotropic AR localization has an impact on the efficacy of anti-AR agents in MBC, need to be explored in future trials.

Abbreviations

AR: Androgen receptor; BC: Breast cancer; CK: Cytokeratin; CTC: Circulating tumour cell; DAPI: 4′6-diamidino-2-phenylindole; EpCAM: Epithelial cell adhesion molecule; FITC: Fluorescein isothiocyanate; HR: Hormone receptor; MBC: Metastatic breast cancer; PB : Peripheral blood; PBMC: Peripheral blood mononuclear cells; TNBC: Triple negative breast cancer

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Authors' contributions

NK coordinated the study, performed the data analysis and drafted the manuscript. NM designed and performed the experiments, collected the data and helped to draft the manuscript. FMS, NH and ND coordinate the study, made substantial contribution to interpretation of the data and reviewed the manuscript. ER, SM, JH, TK, WJ were involved in collection of the data, drafting the manuscript or revising it. MBP, PR, IE made a substantial contribution to interpretation of the data and revised the manuscript. TF designed the study made substantial contribution to interpretation of the data and critically revised the manuscript. All authors read and approved the final manuscript.

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none.

Availability of data and materials

The data that support the findings of this study are available from Tanja Fehm but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Tanja Fehm.

Ethics approval and consent to participate

Written informed consent to participate was obtained from all patients. The study was approved by the Ethical Committee of the Eberhard Karls University of Tuebingen (responsible for DETECT III: 525/2011AMG1) and the local Ethical Committee of the Heinrich Heine University of Duesseldorf (DETECT III: MC-531; DETECT IV: MC-LKP-668).

Consent for publication

This manuscript does not contain any details, images, or videos that might leed to identification of an individual patient. A written informed consent to publish the results od the study -without identifying any participants-was obtained from all the patients.

Competing interests

The authors declare that there are no conflicts of interest.

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The SOX2 Status of Disseminated Tumor Cells in Breast Cancer Patients Treated With Neoadjuvant Chemotherapy

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Abstract. *Background/Aim: Detection of disseminated tumor cells (DTCs) after systemic treatment predicts poor prognosis in breast cancer patients. The aim of our study was to assess the expression of stem-cell marker SOX2 on DTCs and in the primary tumor of patients treated with neoadjuvant chemotherapy (NAT). Materials and Methods: In 170 DTC-positive patients after NAT an additional slide of bone marrow aspirate was stained by double immunofluorescence to detect SOX2-positive DTCs. The SOX2 status of the primary tumor was assessed using the same antibody. Results: The SOX2-status of DTCs was determined in 62 patients and 20 of those (32%) had SOX2 positive DTCs. The SOX2 status of DTCs was not associated with any of the clinicopathological factors. A total of 36% of the patients with a SOX2-negative tumor showed SOX2-positive persistent DTCs. Conclusion: SOX2-positive DTCs can be detected in breast cancer patients after NAT, even in patients with SOX2-negative primary tumors. This suggests that these populations may have evolved independently of each other.*

In the past decade, neoadjuvant therapy (NAT) has become a standard approach in breast cancer (BC) management and is recommended if chemotherapy is indicated based on clinical characteristics and tumor subtype (1). The original aim of NAT was to reduce the size of inoperable or large tumors, thus allowing complete surgical removal and, in some cases, breast conservation (2). However, potential advantages of NAT reach beyond tumor size reduction and include *in vivo* evaluation of tumor sensitivity and identification of non-responders, who can be spared of the unnecessary toxicity of ineffective therapy (3, 4). Moreover, residual tumor burden after NAT is an indicator of unfavourable outcome in most subtypes of BC and may guide the choice of further post-neoadjuvant treatment strategies (5, 6).

While NAT can induce a pathological complete response (pCR) in up to 60% of BC patients, predicting the long-term survival benefit, a relevant proportion of BC patients still suffer from distant recurrence during follow up (6). The presumed pathophysiology of metastatic relapse is based on an early haematogenous spread of cells from the primary tumor. These isolated tumor cells can be detected in peripheral blood (circulating tumor cells, CTCs) or bone marrow (BM) (disseminated tumor cells, DTCs) of patients with most solid malignancies. In breast cancer, presence of CTCs and DTCs has been confirmed as an independent unfavourable prognostic factor for overall survival (OS) and disease-free survival (DFS) (7-11). CTCs/DTCs are nowadays assumed to be a surrogate marker for minimal residual disease (MRD) and their eradication is one of key goals of systemic treatment in non-metastatic BC (12, 13). Since DTCs can persist in secondary homing sites after completion of cytotoxic treatment, their further characterisation aiming at identifying new therapeutic targets is of high clinical interest.

There is a growing body of evidence that tumor progression and metastasis formation can be traced to a small subpopulation of tumor cells with stem-like features, usually

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referred to as cancer stem cells (CSCs) (14, 15). Several studies have shown that these cancer-initiating or stem-like cells persist beyond treatment with cytotoxic agents, suggesting the development of effective mechanisms of chemoresistance (16-19). In this context, it has been hypothesized that at least some DTCs are in fact CSCs. Several studies reported that DTCs with stem-like phenotypes can be detected in the BM of primary BC patients (20-22). Moreover, the presence of stem-like DTCs was shown to predict unfavourable prognosis (22). However, the stem-like features of DTCs persisting beyond neoadjuvant chemotherapy have been scarcely investigated so far (21, 22).

Sex-determining region Y (SRY)-Box2 (SOX2) is a key member of the SOX transcription factor family and an essential embryonic stem cell marker able to induce pluripotency in human somatic cells (23, 24). An important role of SOX2 as a stem cell marker in different human malignancies including breast cancer has been reported previously (25-29). A high expression of SOX2 has also been demonstrated in breast cancer cells that have acquired chemoresistance (30). The aim of this study was to assess the expression of SOX2 in DTCs persistent after NAT in a large cohort of patients with primary non-metastatic breast cancer and to compare it with clinicopathological factors as well as the SOX2 status of the primary tumor.

Patients and Methods

A total of 170 primary breast cancer patients treated from 2001 to 2011 at the Department of Obstetrics and Gynecology, University of Tuebingen, Germany were eligible for this analysis. Non-metastatic BC (T1-T4, N0-3, M0) patients, who received intraoperative BM biopsy and were DTC-positive after completion of NAT were included into the study. Patients with history of any malignancy were excluded. This study was approved by the Ethical Committee of the University of Tuebingen (307/2012R). Patient characteristics are shown in Table I. Pathological complete response (pCR) was defined as the absence of residual invasive cancer in the breast and negative lymph node status after NAT (ypT0/ypTis ypN0). The flow chart of the study is shown in Figure 1.

Collection and analysis of bone marrow. Between 10 and 20 ml of BM were aspirated intraoperatively from the anterior iliac crest under general anaesthesia and processed within 24 hours. All specimens were obtained after written informed consent from patients. This study was approved by the local ethical committee (307/2012R). BM samples were separated by density centrifugation over Ficoll (Biochrom, Germany) with a density of 1.077 g/ml. If necessary, red blood cells were lysed with lysis buffer (155 mM NH4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2). Using a cytocentrifuge (Hettich, Tuttlingen, Germany), 1×10^6 mononuclear cells were spun onto a glass slide and dried at the room temperature, overnight. For each patient, 2×10^6 cells were analyzed and the remaining slides were stored at –20˚C. Slides were than fixed in a 0.5% neutral buffered formalin solution for 10 min and were rinsed in phosphate-buffered saline. Automatic immunostaining was performed on the DAKO autostainer using the monoclonal mouse A45–B/B3 Pan-cytokeratin antibody (Micromet, Munich, Germany), and the DAKO-APAAP detection kit (DakoCytomation, Glostrup, Denmark) according to the manufacturers' instructions. Slides were automatically scanned using the ACIS™ imaging system (ChromaVision, Medical Systems Inc., San Juan, Capistrano, CA, USA) and evaluated based on the recommendations for standardized tumor cell detection as described previously (31, 32). In a subset of DTC-positive patients one additional slide per patient was analysed by immunofluorescence double staining for the presence of SOX2-positive DTCs (1×10^6 cells per patient). Control cytospins with SOX2-positive HT-29 cells were prepared, stored and fixed in the same way.

Immunofluorescence staining of SOX2. One additional slide was thawed at room temperature in a humid chamber for approximately 20 min. After an initial washing step with PBS (Sigma, Munich, Germany), cells were permeabilized with 0.1% Triton X-100 for 12 min and after being washed three times, blocked with normal donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:10 dilution for 30 min. The automated double immunofluorescence staining procedure was performed on the DAKO Autostainer using the polyclonal goat Sox2-antibody (R&D Systems, Inc., Minneapolis, MN, USA) at a 1:50 dilution for 60 min. Cytospins were simultaneously incubated with fluorescein isothiocyanate (FITC) conjugated pan-cytokeratin antibody (C11) (1:500, Sigma, Munich, Germany) for 30 min. Secondary detection was performed with a donkey anti-goat antibody, labelled with Alexa Fluor 594 (1:400, Invitrogen Molecular Probes, Carlsbad, CA, USA) for 30 min. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was used to stain nuclei. Preparations of the colorectal cancer cell line HT-29 mixed with PBMCs from a healthy volunteer served as a positive control for CK and SOX2 staining. For the SOX2 negative control, all conditions were kept the same, except that the primary antibody was omitted. Additionally, cytospins of PBMCs with no added tumor cells served as a negative control for both. Positive and negative control staining is demonstrated in Figure 2.

Fluorescence microscopy. Slides were manually analysed for the presence of tumor cells using a computerised fluorescence microscope Axioplan 2 (×40 oil immersion objectives, Carl Zeiss Micro Imaging GmbH, Göttingen, Germany). To screen for SOX2-positive DTCs a single-pass filter for individual fluorochromes, FITC, Texas Red or DAPI, and a triple-pass filter for (FITC/TRITC/DAPI) were used. Immunostained cells were evaluated based on the morphological criteria of the International Society of Hematotherapy and Graft Engineering Working group for standardisation of tumor cell detection and the consensus statements (33, 34). Cytokeratin-positive cells that contained an intact nucleus (DAPI positive) were identified as DTCs. DTCs with either moderate or intense staining of the nucleus were considered SOX2 positive. Slides were evaluated by two, or in doubtful cases, three independent investigators (TF, KJ and HN).

Immunohistochemical staining of the primary tumor. Immunohistochemical analysis was performed either on core biopsies or surgical resection specimens according to the method described previously by our group (35). Staining was performed on 3 to 5 μm thick sections using DAB Map Detection Kit and heat-induced antigen retrieval (HIER). The polyclonal goat SOX2 antibody (R&D Systems, Inc.) was diluted 1:40 in DISCOVERY Antibody Diluent

Table I. *Clinical data of all patients included into the trial.*

	n N=170 (%)
Total	170
Menopausal status	
Premenopausal	86 (51)
Postmenopausal	84 (49)
Tumour size before NAT	
cT1	2 (1)
cT2	82 (48)
cT3	43 (25)
cT4	38 (22)
unknown	5 (3)
Tumor size after NAT	
ypT0/ypTis	44 (26)
ypT1	66 (39)
ypT2-4	60 (35)
Nodal status before NAT	
Negative	49 (29)
Positive	117 (69)
Unknown	4 (2)
Nodal status after NAT	
ypN0	89 (52)
ypN+	81 (48)
Pathologic response	
pCR	38 (22)
non-pCR	132 (78)
Histology	
Ductal	141 (83)
Lobular	26 (15)
Others	3 (2)
Grading	
I/II	114 (67)
III	56 (33)
ER status	
Negative	73 (43)
Positive	97 (57)
PR status	
Negative	46 (27)
Positive	124 (73)
HER2 status	
Negative	133 (78)
Positive	37 (22)
IHC subtype	
HR+/HER2–	92 (54)
HR+/HER2+	19 (11)
HR–/HER2+	18 (11)
TNBC	41 (24)

ER: Estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; IHC: immunohistochemistry; TNBC: triple negative breast cancer, NAT: neoadjuvant treatment; pCR: pathological complete response.

(Ventana) and applied according to the manufacturer's instructions. Secondary detection was performed with a rabbit anti-goat antibody (Jackson ImmunoResearch, Inc., West Grove, PA, USA) at a 1:200 dilution. 3,3'diaminobenzidine (DAB) was used as a chromogen. Finally, the slides were counterstained with haematoxylin and mounted for examination. For assessment of the SOX2 status, the percentage of cells with nuclear reactivity (score 0: none, 1: >0% <10%, 2: >10% < 50%, 3: ≥50% <90%, 4: ≥90%) was determined according to the score published by Pham *et al*. (36). Tumors with a score of 1 or more were considered SOX2 positive.

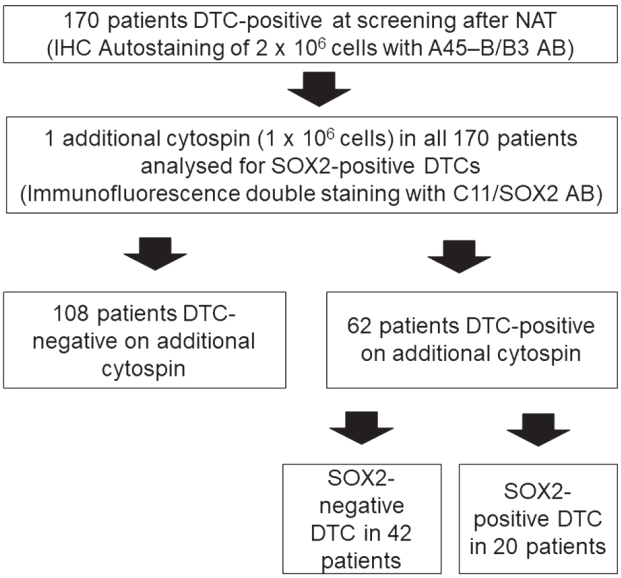

Figure 1. *Study flow chart. DTC: Disseminated tumor cell, AB: antibody, IHC: immunocytochemistry, NAT: neoadjuvant therapy.*

Statistical analysis. A chi-squared test was used to evaluate the relation between SOX2-positive DTCs and/or primary tumor and clinicopathological factors. Statistical analysis was performed by SPSS, version 24 (SPSS Inc., Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

Results

Patients' characteristics. A total of 170 primary BC patients were included in the analysis. The clinical data of patients are listed in Table I. 86 out of 170 (51%) patients were premenopausal. The most common histological tumor type was invasive ductal carcinoma (83%). Estrogen and progesterone receptor (ER, PR) status were positive in 57% and 73% of these patients, respectively. 37 patients (22%) had HER2-positive tumors. All patients were treated with NAT. 38 out of 170 (22%) patients achieved pathological complete response (pCR).

SOX2 status of DTCs after NAT. SOX2 status of persistent DTCs was determined in 62 patients after NAT. Among these 62 patients, SOX2-positive DTCs were detected in 20 cases (32%; Table II, Figure 3). No significant correlation was observed between SOX2 status of DTCs and any

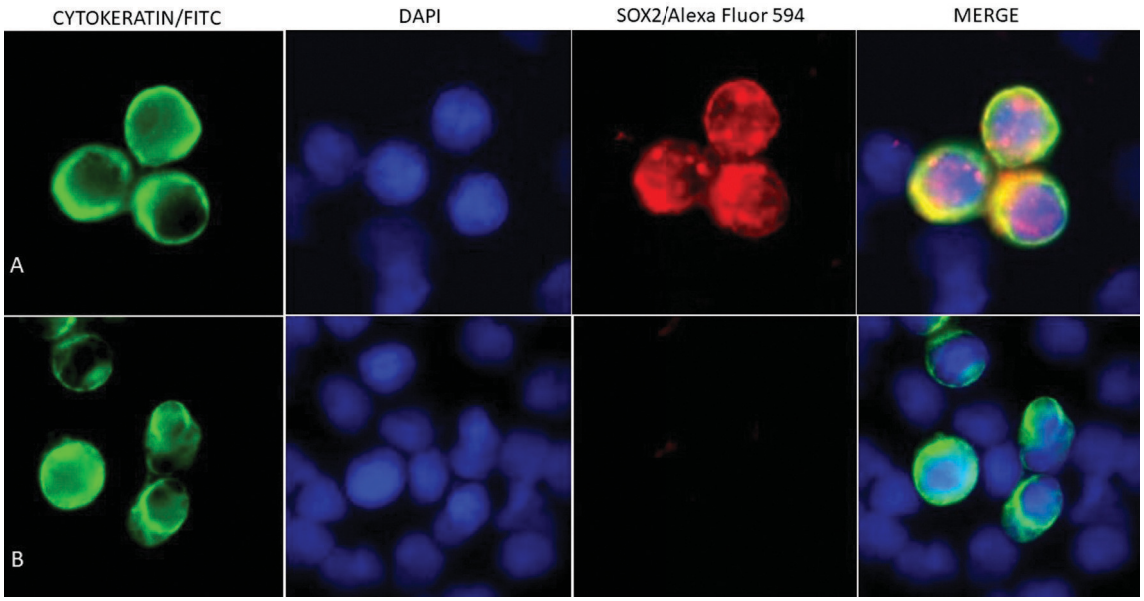

Figure 2. *SOX2 control staining. (A) Positive control staining (HT-29 cells). (B) Negative control staining (HT-29 cells, primary antibody omitted) (×63 oil immersion objective).*

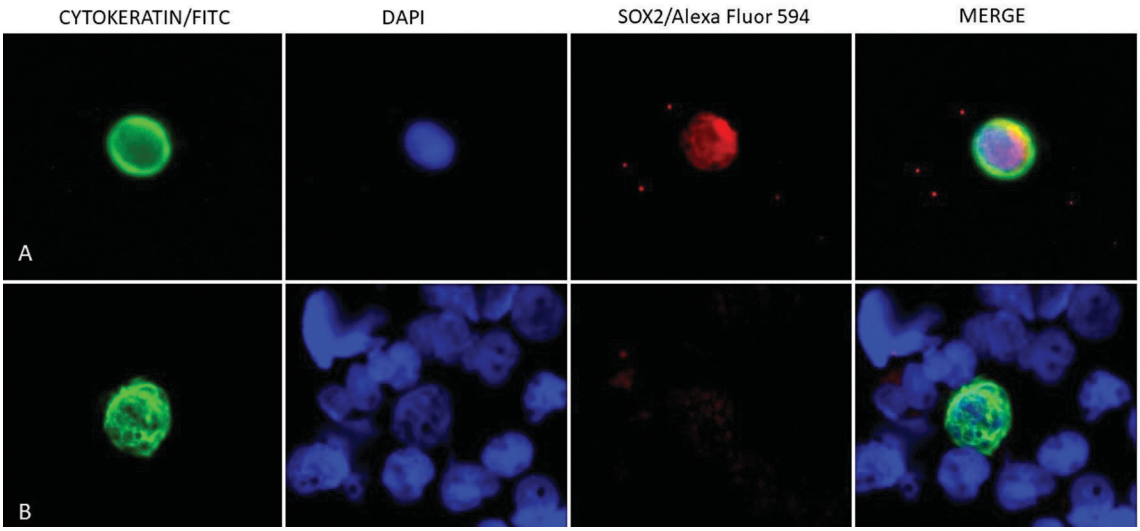

Figure 3. *SOX2 staining of DTCs in primary breast cancer patients. (A) SOX2-positive DTC. (B) SOX2-negative DTC (×63 oil immersion objective).*

clinicopathological characteristics. SOX2 status of DTCs persisting beyond NAT was not associated with pathological response to treatment.

Expression of SOX2 in the primary tumor. Primary tumor tissue was available for immunohistochemical determination of SOX2 status in 38 patients prior to systemic treatment and in 27 patients after NAT. The tumors were SOX2-negative in most cases [30 out of 38 (79%) before and 18/27 (67%) after NAT, respectively]. No correlation could be found between the SOX2 status of primary tumor (pre- and post-therapeutic) and any of the established prognostic factors. Neither was the SOX2 status of primary tumor associated with response to NAT. In 17 patients, the SOX2 status has been assessed on persistent DTCs and the primary tumor

Table II. *Clinical data of 62 patients included in further analysis of SOX2-status of DTC.*

	n (%)	SOX2-positive DTCs (%)	*p*-Value*
Total	62 (100)	20 (32)	
Menopausal status			1.0
Premenopausal	31 (50)	10 (32)	
Postmenopausal	31 (50)	10 (32)	
Tumor size before NAT			0.76
cT1	1 (2)	0 (0)	
cT2	27 (43)	8 (30)	
cT3	13 (21)	6 (46)	
cT4	18 (29)	5 (28)	
unknown	3 (5)	1 (33)	
Tumor size after NAT			0.45
ypT0/ypTis	16 (26)	4 (25)	
ypT1	24 (39)	10 (42)	
ypT2-4	22 (35)	6 (27)	
Nodal status before NAT			0.94
Negative	14 (23)	4 (29)	
Positive	45 (73)	15 (33)	
Unknown	3 (5)	1 (33)	
Nodal status after NAT			0.47
ypN0	30 (48)	11 (37)	
ypN+	32 (52)	9 (28)	
Pathologic response			0.55
pCR	12 (19)	3 (15)	
non-PCR	50 (81)	17(27)	
Histology			0.53
Ductal	52 (84)	18 (35)	
Lobular	8 (13)	2 (25)	
Others	2 (3)	0 (0)	
Grading			0.95
I/II	40 (65)	13 (32)	
III	22 (35)	7 (32)	
ER status			0.79
Negative	20 (32)	6 (30)	
Positive	42 (68)	14 (33)	
PR status			0.59
Negative	13 (21)	5 (38)	
Positive	49 (79)	15 (31)	
HER2 status			0.36
Negative	45 (73)	13 (29)	
Positive	17 (37)	7 (41)	
IHC subtype			0.22
HR+/HER2–	34 (55)	10 (29)	
HR+/HER2+	11 (18)	5 (45)	
HR–/HER2+	6 (10)	2 (33)	
TNBC	11 (18)	3 (27)	

*Chi-squared test. ER: Estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; IHC: immunohistochemistry; TNBC: triple negative breast cancer; NAT: neoadjuvant treatment; pCR: pathological complete response.

before systemic treatment and showed a concordance rate of 59% (Table III). The SOX status of the primary tumor before and after NAT was evaluated in 18 patients and was concordant in 78% of cases (p=0.045, Table IV).

Discussion

Disseminated tumor cells persisting beyond cytotoxic treatment predict impaired survival in primary breast cancer patients (10, 11, 37). These cells are currently assumed to serve as a surrogate marker of minimal residual disease and their eradication is considered to be a main target of systemic therapy. However, about a half of DTC-positive BC patients remain tumor-free during a follow up period of over 10 years (7, 38). This phenomenon may be explained by the so-called "metastatic inefficiency". According to this hypothesis, only a small population of DTCs is able to persist and subsequently cause tumor growth in secondary sites (39, 40). One theory presently under discussion is the hypothesis that some of these cells undergo the process of epithelial-mesenchymal transition (EMT) that increases their invasiveness and leads to acquisition of stem-cell features (17, 41, 42). These cancer stem cells can evade systemic treatment and are thought to play a major role in the metastasis cascade (18, 42). In this context, we assessed the expression of the stem cell marker SOX2 on DTCs persisting in the BM of BC patients after NAT.

In 170 patients with persistent DTCs after completion of neoadjuvant therapy, an additional bone marrow cytospin was analyzed. In 62 cases, at least one DTC could be found and these patients were included in further analysis of the SOX2 status. Why some of the additional cytospins contained no DTCs can be explained by several factors, such as the freezing and thawing process of the slides, staining of only one additional slide (1×10^6 cells per patient) compared to two slides (2×10^6 cells per patient) analyzed in the routine IHC staining as well as different assays (IHC *vs.* immunofluorescence) and the different anticytokeratin anibodies used (A45-B/B3 *vs.* C11).

To assess SOX2 status on persistent DTCs, we developed a double immunofluorescence staining assay based on cytokeratin positivity and morphological criteria according to the Consensus Recommendations for Standardized Tumor Cell Detection (34). 32% of DTC-positive patients had at least one SOX2 positive tumor cell in BM. This is, to the best of our knowledge, the largest study demonstrating that DTCs persistent after NAT express a stem cell associated feature and the first evaluating SOX2 expression on DTCs in BC patients. Reuben *et al*. have analyzed DTCs in 30 BC patients after NAT in terms of stemness and found epithelial CD44+CD24low cells in 57% of these patients (21). Similar to our observations, a detection of potential CSC in BM was not associated with response to NAT. In another study by Giordano *et al*., 18 of 26 patients (69%) had potential CSCs in BM after NAT (22). The same detection method, a multi-parameter flow cytometry, was used in both trials (21, 22) which might explain the much higher CSC positivity rates compared to our study. Further, both trials used

Table III. *SOX2 status of persistent DTCs and primary tumor before NAT.*

	SOX2-status	DTC		Total (%)
		SOX2 negative (%)	SOX2 positive (%)	
PT	SOX2 negative (%)	9 (53)	5 (29)	14 (82)
	SOX2 positive (%)	2 (12)	1 (6)	3 (18)
	Total (%)	11 (65)	6 (35)	17 (100)

PT: Primary tumor; DTC: disseminated tumor cell; NAT: neoadjuvant treatment.

Table IV. *SOX2 status of the primary tumor before and after NAT.*

	SOX2-status	PT pre-NAT		Total (%)
		SOX2 negative (%)	SOX2 positive (%)	
PT post-NAT	SOX2 negative (%)	11 (61)	3 (17)	14 (78)
	SOX2 positive (%)	1 (5.6)	3 (17)	4 (22)
	Total (%)	12 (66)	6 (34)	18 (100)

PT: Primary tumor; NAT: neoadjuvant treatment.

ALDH/CD44/CD24 and not SOX2 as CSC marker, making a direct comparison of our studies difficult.

Our analysis demonstrates that some of the cells detected in secondary homing sites after NAT may exhibit a stem-like phenotype. Tumor initiating-capacity on the one hand and ability to elude cytotoxic therapy and persist in a quiescent and/or dormant state on the other hand, are the features postulated to account for chemoresistance and metastatic potential of CSCs (43). A high expression of SOX2 has been indeed demonstrated in BC cell lines known for their cross-resistance to taxanes, anthracyclines and cisplatin (30). Furthermore, SOX2 expression has been linked to tamoxifen resistance in BC (44) and was shown to significantly affect adhesion properties of BC cells (45). SOX2 was also recently shown to mediate proliferation and dissemination in lung cancer cells resistant to tyrosine kinase inhibitors (46). The CSC hypothesis is supported by the phenomenon of tumor cell dormancy, clinically well-known in BC patients, who can experience a relapse after a very long period, sometimes up to 25 years, without evidence of the disease (47, 48). In concordance with this clinical observation isolated tumor cells have been detected in the blood of asymptomatic BC patients up to 22 years after primary surgery (49). However, these persistent cells have not been analyzed in terms of stem cell-like features in any of the available studies.

While studies on the expression of SOX2 on DTCs are missing, data on the SOX2 expression in primary BC tissue have been reported previously (35, 50) In our cohort, 21% of patients have SOX2-positive tumors prior to NAT and 33% of tumors were SOX2-positive after NAT. This is in line with our earlier analysis demonstrating a SOX2 tumor positivity rate of 28% (24/86 patients) (35), compared to 16.7% (33/198 patients) reported by Rodriguez-Pinilla *et al*. (50). The fact that the SOX2 positivity rate of the primary tumor in our cohort was higher after NAT than prior to the systemic therapy is consistent with the reported phenomenon that CSC frequency increases in BC tissue after cytotoxic treatment (18, 51). Recently, chemotherapy was shown to induce BC stemness in a xenograft mouse model (52). A direct comparison of SOX2 status between pre- and post-therapeutic tumor tissue was possible in 18 patients (Table IV). In this group, the SOX2 status remained the same in most patients, with only one patient acquiring SOX2 positivity and three patients converting from positive to negative SOX2 status.

In 41% of analyzed patients, the SOX2 status of primary tumor before NAT differed from the SOX2 status of persistent DTCs. A positive SOX2 status of DTCs was observed in 36% of patients (5 of 14 cases) with SOX2-negative tumors (Table III). A discrepancy between tumor and (persistent) DTCs regarding other phenotypic features has been described in previous studies (53-55), showing that MRD cells may evolve independently from the primary tumor. This observation is consistent with the parallel tumor progression model proposed by Klein *et al*. (56).

Another aspect evaluated in our study was the correlation of SOX2 status of primary tumors/DTCs and other clinical-pathological factors. Previously published studies reported a significant association between SOX2 positivity and higher grading, nodal positivity and poor prognosis (57-59). In contrast, no correlations were observed in our study, possibly due to the fact that the SOX2 status of both the tumor and DTCs was only available in a small proportion of patients.

Limitations of our Study

Even though DTC detection based on their epithelial and morphological features is considered standard, the lack of single-cell molecular analysis confirming tumor origin and SOX2-positivity of these cells at the genomic level may be considered a potential limitation of our study. Furthermore, analysis of a whole BM suspension (approx. 5-10 ml), aspirated from each patient instead of one cytospin with 1×10^6 cells per patient would possibly provide higher DTC numbers and result in higher numbers of DTCs available for analysis of the SOX2 status. Further trials implementing molecular characterization of single DTCs as well as analysis of other stem cell-associated markers are necessary to confirm the stem-like character and to establish the DTCs' clinical relevance.

Conclusion

In the present study, we demonstrated that DTCs with stem-like phenotype can persist after neoadjuvant treatment in a relevant number of breast cancer patients. SOX2-positive DTCs were detected in patients with SOX2-negative primary tumors, suggesting that these populations may have evolved independently of each other. Stem-like character of minimal residual disease should be further evaluated using molecular analyses in future studies.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

NK performed the data analysis and drafted the manuscript; KJ performed the IF experiments and collected the data; MBP made a substantial contribution to interpretation of the data and helped to draft the manuscript; AS performed and evaluated the IHC experiments of the primary tumors; HN coordinated the study and reviewed the manuscript; TF designed the study, made substantial contributions to interpretation of the data and reviewed the manuscript; HA, BS, WD and MC were involved in interpretation of the data, drafting of the manuscript or revising it. All Authors read and approved the final manuscript.

Availability of Data and Materials

The data that support the findings of this study are available from the authors upon reasonable request and with permission from Tanja Fehm.

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Invasive Breast Carcinoma with Neuroendocrine Differentiation: A Single-Center Analysis of Clinical Features and Prognosis

Invasives Mammakarzinom mit neuroendokriner Differenzierung: eine monozentrische Analyse der klinischen Merkmale und Prognose

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Key words

neuroendocrine neoplasia of the breast, invasive breast cancer with neuroendocrine differentiation, neuroendocrine breast cancer, neuroendocrine markers, somatostatin receptor 2A

Schlüsselwörter

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ABSTRACT

Introduction Invasive breast cancer with neuroendocrine differentiation is a rare subtype of breast malignancy. Due to frequent changes in the definition of these lesions, the correct diagnosis, estimation of exact prevalence, and clinical behaviour of this entity may be challenging. The aim of this study was to evaluate the prevalence, clinical features, and outcomes in a large cohort of patients with breast cancer with neuroendocrine differentiation.

Patients Twenty-seven cases of breast cancer with neuroendocrine differentiation have been included in this analysis. Twenty-one cases were identified by systematic immunohistochemical re-evaluation of 465 breast cancer specimens using the neuroendocrine markers chromogranin A and synaptophysin, resulting in a prevalence of 4.5%. A further six cases were identified by a review of clinical records.

Results Median age at the time of diagnosis was 61 years. 70% of patients had T2–4 tumors and 37% were node-positive. The most common immunohistochemical subtype was HR-positive/HER2-negative (85%). 93% were positive for synaptophysin and 48% for chromogranin A. Somatostatin receptor type 2A status was positive in 12 of 24 analyzed tumors

(50%). Neuroendocrine-specific treatment with somatostatin analogues was administered in two patients. The 5-year survival rate was 70%.

Conclusions Breast cancer with neuroendocrine differentiation is mostly HR-positive/HER2-negative and the diagnosis is made at a higher TNM stage than in patients with conventional invasive breast carcinoma. Moreover, breast cancer with neuroendocrine differentiation was found to be associated with impaired prognosis in several retrospective trials. Due to somatostatin receptor 2A expression, somatostatin receptor-based imaging can be used and somatostatin receptor-targeted therapy can be offered in selected cases.

ZUSAMMENFASSUNG

Einleitung Invasives Mammakarzinom mit neuroendokriner Differenzierung ist eine seltene Unterart von Brustkrebs. Da die Definition dieser Läsionen häufig geändert wurde, kann eine korrekte Diagnose sowie eine richtige Einschätzung der genauen Prävalenz und des klinischen Verhaltens dieser Entität Schwierigkeiten bereiten. Ziel dieser Studie war es, die Prävalenz, die klinischen Merkmale und das Outcome in einem großen Patientenkollektiv von Frauen mit Mammakarzinom und neuroendokriner Differenzierung zu evaluieren.

Patientinnen Die Daten von 27 Patientinnen mit Brustkrebs mit neuroendokriner Differenzierung wurden in diese Analyse aufgenommen. 21 Fälle wurden durch eine systematische immunohistochemische Reevaluierung von 465 Brustkrebsproben mit Verwendung der neuroendokrinen Basismarker Chromogranin A und Synaptophysin identifiziert, was einer Prävalenz von 4,5% entspricht. Sechs weitere Fälle wurden durch eine Überprüfung der klinischen Krankenakten indentifiziert.

Ergebnisse Das durchschnittliche Alter zum Zeitpunkt der Diagnose betrug 61 Jahre. 70% der Patientinnen hatten T2–4 Tumoren, und 37% hatten positive Lymphknotenbefunde. Die häufigste immunohistochemische Unterart war HR-positiv/HER2-negativ (85%). 93% waren für Synaptophysin und 48% für Chromogranin A positiv. Der Somatostatin-Rezeptor-2A-Status war in 12 von 24 analysierten Tumoren positiv (50%). Zwei Patientinnen erhielten eine neuroendokrin-spezifische Therapie mit Somatostatin-Analoga. Die 5-Jahres-Überlebensrate betrug 70%.

Schlussfolgerungen Brustkrebs mit neuroendokriner Differenzierung ist meist HR-positiv/HER2-negativ, und die Diagnose wird meist in einem höheren TNM-Stadium gestellt als bei Patientinnen mit herkömmlichem invasiven Mammakarzinom. Darüber hinaus war der Brustkrebs mit neuroendokriner Differenzierung in mehreren retrospektiven Studien mit einer schlechten Prognose assoziiert. Im Falle eines positiven SSTR2A-Status kann eine Somatostatin-Rezeptor-basierte Bildgebung eingesetzt werden, und in ausgewählten Fällen eine zielgerichtete Therapie mit Somatostatinanaloga angeboten werden.

Abbreviations

BC-NE	breast cancer with neuroendocrine differentiation
BC-NST	breast cancer no special type
LCNEC	large cell neuroendocrine cancer
NE	neuroendocrine
NET	neuroendocrine tumor
NEN	neuroendocrine neoplasia
SCNEC	small cell neuroendocrine cancer
SSA	somatostatin analogues
SSTR	somatostatin receptor

Background

Primary neuroendocrine neoplasia (NEN) of the breast is a rare subtype of breast cancer (BC) representing < 1% of all NENs, which occur most commonly in the gastrointestinal tract and the lung [1,2]. The prevalence of neuroendocrine differentiation among BC patients varies between 0.1 and 20% in the literature, with the World Health Organization (WHO) reporting a prevalence of up to 5% of BC cases [3]. This discrepancy is due to the fact that the diagnostic criteria and definition of this heterogeneous group of lesions have frequently changed in the last two decades, and neuroendocrine immunohistochemical markers are not routinely used in BC diagnostics [4]. The previous and current WHO classification of NEN of the breast are shown in ▶ **Table 1**.

Neuroendocrine differentiation in BC was first described by Feyrter and Hartmann in 1963; this was followed by a series of eight patients with “primary carcinoid tumor of the breast” reported by Cubilla and Woodruff in 1977 [5,6]. Since then, many authors have tried to describe and characterize this heterogeneous entity until in 2000, Sapino et al. proposed a definition for NEN of the breast as a subset of tumors with specific morphological features and expression of the neuroendocrine markers chromogranin and/or synaptophysin in more than 50% of tumor cells [7]. This definition was later adopted by the WHO classification of NEN of the breast introduced in 2003 and last modified in 2019 [8–10].

While earlier classifications included a category comprising a subset of BC (no special or special type, e.g., mucinous, papillary etc.) with neuroendocrine differentiation as determined by morphological and immunohistochemical analysis, the latest version excludes BC-NE from the NEN group altogether (▶ **Table 1**). Through these changes, the WHO has attempted to develop a uniform classification framework for NENs at different anatomical sites to provide pathologists and clinicians with a consistent management strategy for NEN patients, since neuroendocrine differentiation in BC, with the exception of small cell carcinoma, is assumed to have no therapeutic significance [3].

However, there are certain diagnostic and therapeutic aspects of BC-NE that should be acknowledged, even if current guidelines recommend treatment based on the general principles of breast cancer therapy. The aims of this retrospective study were:

1. to analyze the clinical features and treatment strategies of BC-NE,
2. to assess the prognostic impact of BC-NE, and
3. to compare our results to previously published studies.

▶ **Table 1** Different classifications of NEN of the breast in the last two decades.

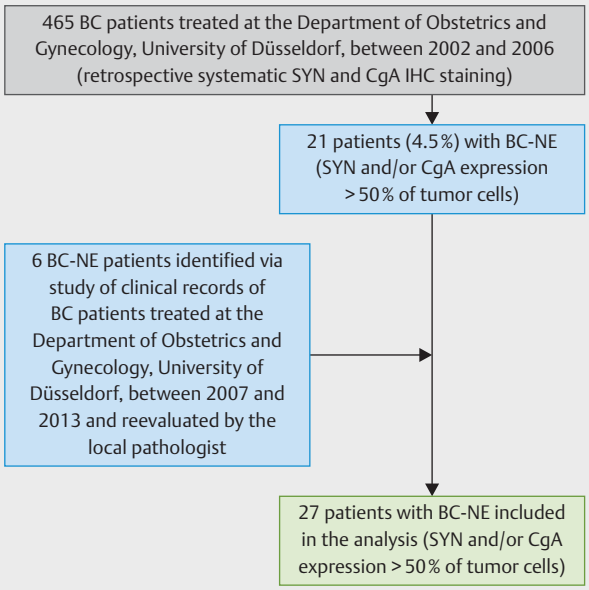
WHO 2003 [8]	WHO 2012 [9]	WHO 2019 [10]
Solid neuroendocrine carcinoma (NEC)*	Well differentiated neuroendocrine tumor (WD-NET)[2]	Neuroendocrine tumor (NET) ▪ grade 1 ▪ grade 2
	Invasive breast carcinoma with neuroendocrine differentiation** ▪ special type ▪ no special type	Invasive breast cancer with neuroendocrine differentiation overridden by morphological tumor type should not be classified as a true neuroendocrine neoplasia but as a morphological subtype (e.g., NST, mucinous, papillary) with neuroendocrine differentiation
Large cell neuroendocrine carcinoma (LCNEC)[1]		Large cell neuroendocrine carcinoma[1] (LCNEC)
Small cell/oat cell carcinoma (SCNEC)[1]	Poorly differentiated neuroendocrine carcinoma (PD-NEC)/small cell carcinoma[1]	Small cell neuroendocrine carcinoma[1] (SCNEC)

* Expression of neuroendocrine markers > 50 % (particularly chromogranin A and/or synaptophysin), ** no threshold for the expression of the neuroendocrine markers, [1] analogous to small-cell or large-cell lung cancer, [2] low grade tumors morphologically similar to carcinoid tumors of other sites. NST: no special type.

Materials and Methods

Patient material

A total of 27 patients with BC-NE treated at the Department of Obstetrics and Gynecology of the University of Duesseldorf, Germany, between 2002 and 2013 were included in this analysis. Surgically excised breast specimens from 465 BC patients treated between 2002 and 2006 were systematically re-evaluated in terms of neuroendocrine differentiation. Moreover, a review of the clinical records of BC patients treated at our department between 2007 and 2013 was performed to identify further BC-NE patients. Inclusion criteria were: primary breast cancer with neuroendocrine differentiation (T1–T4, N0–3, M0/M1) (TNM, 8th edition 2017) defined as > 50 % positivity for the immunohistochemical neuroendocrine markers chromogranin A and/or synaptophysin according to the NEN definition from 2003 (▶ **Table 1**). Exclusion criteria were the following entities: poorly differentiated large or small cell neuroendocrine carcinoma and well differentiated neuroendocrine tumor (NET, G1). The flow chart showing patient selection for our analysis is presented in ▶ **Fig. 1**. The study was approved by the local Ethical Committee of the Heinrich Heine University of Duesseldorf (Study number 4524).



▶ **Fig. 1** Flow chart of the selection process. Abbreviations: SYN: synaptophysin, CgA: chromogranin A, BC-NE: invasive breast cancer with neuroendocrine differentiation, IHC: immunohistochemistry.

Immunohistochemistry staining

Tissue sections (2 µm) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxide. Blocking non-specific protein-binding sites, normal mouse serum was applied. Neuroendocrine markers were detected with specific monoclonal mouse antibodies for synaptophysin (NCL-L-Synap 299, Novocastra, Berlin, Germany) and chromogranin A (MAB 5268, Chemikon, Schwalbach, Germany) at a dilution of 1 : 100 and 1 : 1000, respectively. Immunostaining was performed with anti-mouse IgG and Vectastain ABC, followed by chromogen detection. Finally, the slides were counterstained with hematoxylin and mounted for examination. SSTR 2A status was determined with monoclonal rabbit antibody (UMB1, Abcam, Cambridge, UK) at a dilution of 1 : 50. Membranous staining was scored as: 0: no staining; 1: weak staining (< 10 %); 2+: moderate staining (10–80 %); and 3+: strong staining (> 80 % tumor cells).

Statistical analysis

Statistical analysis was performed using SPSS (version 25). Survival intervals were measured from the time of diagnosis until death or the first clinical, radiological or pathological diagnosis of relapse, whichever occurred first. Relapse was defined as either local

▶ **Table 2** Clinicopathological features and administered therapy in the study cohort.

	n (%)
Total	27 (100)
Age at diagnosis	
▪ < 50	4 (15)
▪ 50–69	13 (48)
▪ ≥ 70	10 (37)
Menopausal status	
▪ Premenopausal	5 (18.5)
▪ Postmenopausal	22 (81.5)
Stage at diagnosis	
▪ I	6 (22)
▪ II	14 (52)
▪ III	3 (11)
▪ IV	3 (11)
▪ Unknown	1 (4)
Tumor stage	
▪ T1	7 (26)
▪ T2	16 (60)
▪ T3-4	3 (11)
▪ Unknown	1 (4)
Tumor focality	
▪ Unifocal	21 (78)
▪ Multifocal	5 (19)
▪ Unknown	1 (4)
DCIS component	
▪ Yes	12 (44
▪ No	15 (56)
Nodal status	
▪ Negative	15 (56)
▪ Positive	10 (37)
▪ Unknown	2 (7)
Lymphatic vessel infiltration	
▪ L0	11 (41)
▪ L1	8 (30)
▪ Unknown	8 (30)
Original histology	
▪ NST	16 (59)
▪ Lobular	1 (4)
▪ NST/lobular	1 (4)
▪ Mucinous	4 (15)
▪ NET*	5 (18)
Grading	
▪ II	21 (78)
▪ III	6 (22)
Ki-67 index	
▪ < 15	6 (22)
▪ 15–29	8 (30)
▪ ≥ 30	11 (41)
▪ Unknown	2 (7)
IHC subtype	
▪ HR+/HER2−	23 (85)
▪ HR+/HER2+	2 (7)
▪ HR−/HER2+	0 (0)
▪ TNBC	2 (7)
SSTR-based imaging performed	
▪ Yes	5 (19)
▪ No	22 (81)
Surgical procedure	
▪ Mastectomy	14 (52)
▪ Breast-conserving surgery	11 (41)
▪ None	2 (7)
AT-based Chemotherapy	
▪ Yes	14 (52)
▪ No	13 (48)
Endocrine therapy	
▪ Yes	24 (89)
▪ No	3 (11)
NE-specific therapy	
▪ Yes	2 (7)
▪ No	25 (93)

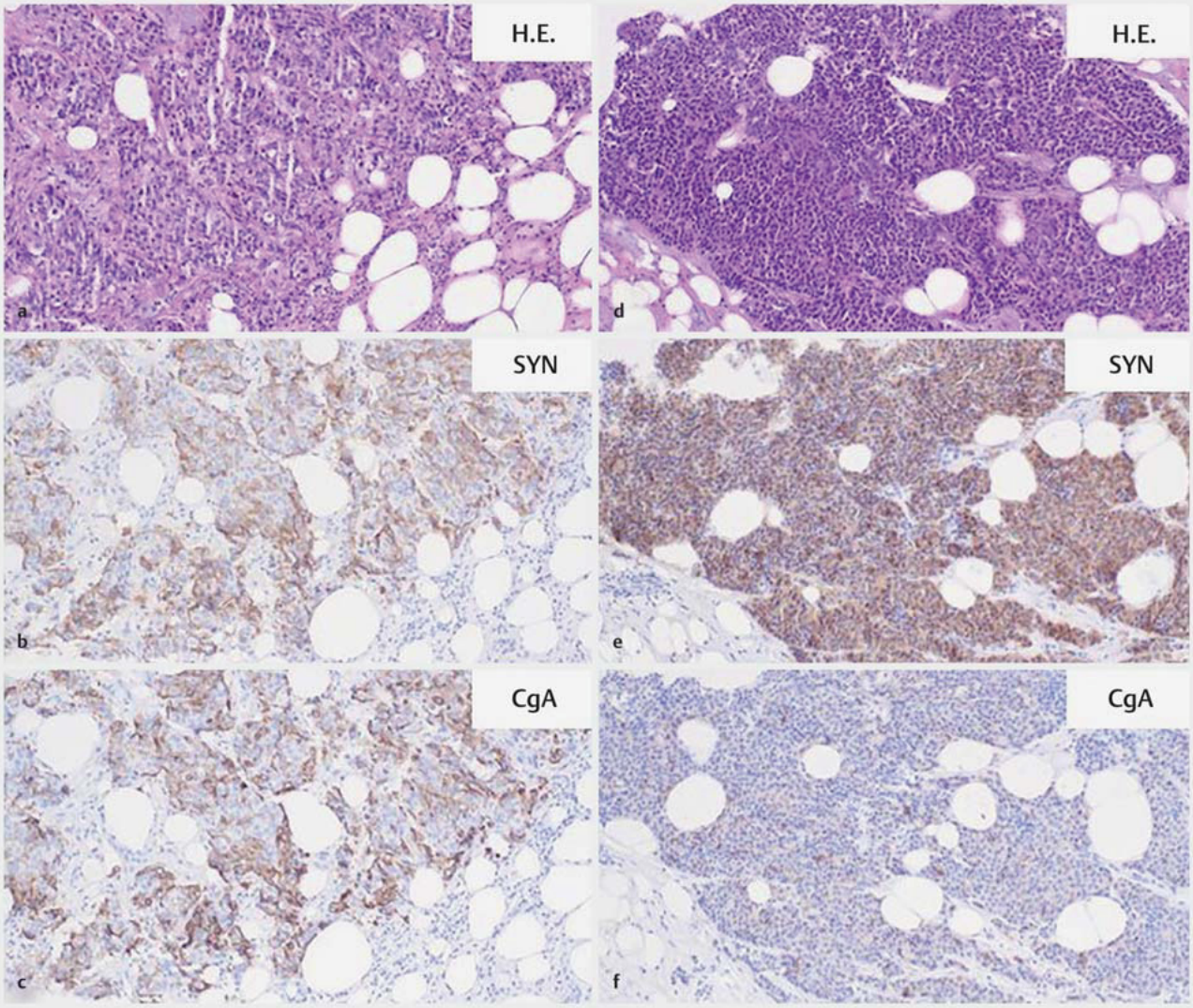
* Initially diagnosed as NET G2. TNBC: triple negative breast cancer, BCS: breast conserving surgery, NE: neuroendocrine, SSTR: somatostatin receptor, AT: anthracycline-taxane. Numbers in parentheses are percentages and do not add to 100 in some instances owing to rounding.

recurrence or distant metastasis. Survival was calculated using the Kaplan-Meier method. Primarily metastatic patients were excluded from the disease-free survival (DFS) analysis.

Results

Patients' characteristics

Clinical data from 27 patients with BC-NE were eligible for this study. Twenty-one of these patients were identified by a systematic immunohistochemical re-evaluation of 465 breast surgical specimens with regard to NE differentiation, resulting in a prevalence of 4.5%. A further six patients were identified through an analysis of the clinical records of BC patients treated between 2007 and 2013 and subsequent histological re-evaluation (▶ **Fig. 1**). Clinical features of the study cohort are presented in ▶ **Table 2**. The median age at the time of diagnosis was 61 years

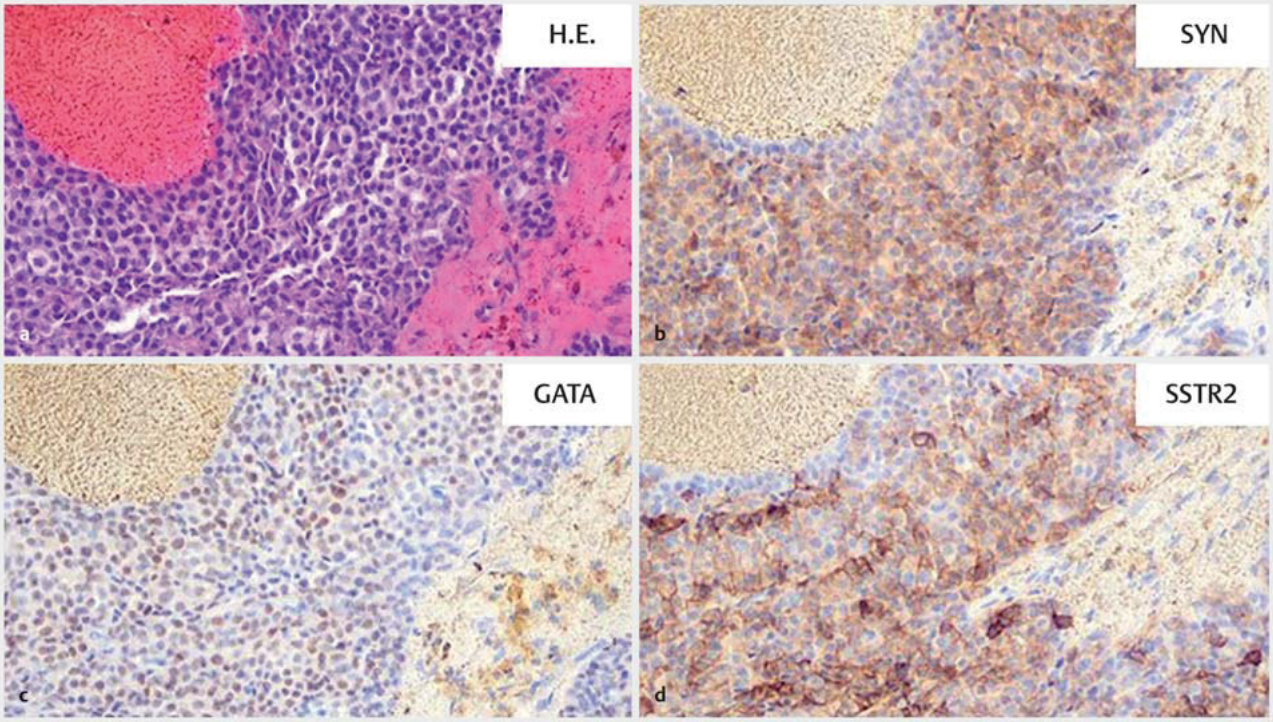


▶ **Fig. 2** Histopathology and expression of general neuroendocrine marker proteins in two different breast carcinomas with neuroendocrine differentiation. **a, d** Hematoxylin and eosin (H. E.) staining demonstrates a solid growth pattern and complete lack of tubular architecture in both carcinomas. Cytology of the tumor cells in **a** show an NST-like pattern, while cytology of the tumor cells in **d** is highly suggestive for a neuroendocrine phenotype. **b, e** Expression of the pan-neuroendocrine marker synaptophysin (SYN) in more than 50 % of tumor cells in **b** and in 100 % of tumor cells in **e. c, f** Expression of the large dense core neuroendocrine vesicle marker chromogranin A (CgA) in more than 50 % of tumor cells in **c**, while tumor cells in **f** are positive in a minor subpopulation.

(range 38–84 years) and 22 out of 27 patients (82 %) were postmenopausal. Nineteen patients (70 %) had T2–4 tumors and 10 (37 %) were node-positive with lymphatic vessel infiltration (L1) detected in 8 out of 27 cases (30 %). The most common immunohistochemical tumor subtype was HR-positive/HER2-negative, diagnosed in 23 patients (85 %), followed by HR-positive/HER2-positive and triple-negative BC in two patients each (7 %). Thirteen tumors (48 %) were positive for chromogranin A (CgA) and 25 (93 %) were positive for synaptophysin (Syn), whereas 12 tumors (44 %) expressed both markers in > 50 % of tumor cells (▶ **Fig. 2**, **Table 3**). Somatostatin receptor type 2A (SSTR 2A) was analyzed in 24 tumors and of which 12 (50 %) showed a SSTR 2A-positive status (▶ **Fig. 3**, **Table 3**). None of the patients in our cohort presented with specific clinical symptoms due to neuroendocrine tumor differentiation.

Clinical diagnosis and treatment

Standard thoracic and abdominal imaging (CT scan or ultrasound and X-ray according to the current recommendations and internal standards) as well as bone scans were performed in all patients at the time of diagnosis to exclude metastatic disease. Additional SSTR-based neuroendocrine imaging (octreoscan or ^{68}Ga-DOTA-TOC PET/CT) was performed in five patients with known neuroendocrine differentiation of BC at the time of the diagnosis and a SSTR-positive score. Two primary metastatic patients received an octreotide scan to confirm the NE differentiation of the metastatic



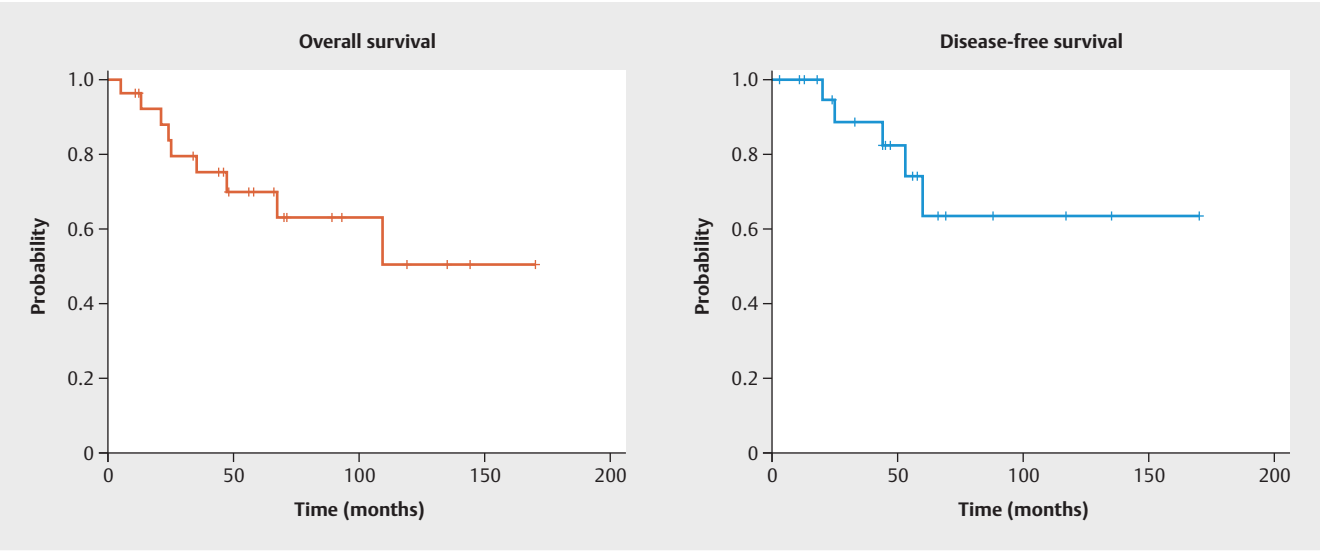
▶ **Fig. 3** Expression of the nuclear transcription factor GATA and the somatostatin receptor 2A in breast carcinoma with neuroendocrine differentiation. **a** Hematoxylin and eosin (H.E.) staining reveals a solid growth pattern, complete lack of tubular architecture and a cytology highly suggestive of neuroendocrine differentiation. **b** Expression of the pan-neuroendocrine marker synaptophysin (SYN) in approximately all tumor cells. **c** Nuclear expression of the breast-specific transcription factor GATA in the majority of tumor cells. **d** Circular membranous staining for the somatostatin receptor type 2A (SSTR 2) in a major subpopulation of tumor cells.

sites. In one patient with diffuse NE bone marrow infiltration and disease progress after chemotherapy with epirubicin weekly and endocrine therapy with fulvestrant, the octreotide scan was performed in order to evaluate the possibility of SSTR-specific radionuclide therapy. This therapy was not administered as the patient's condition worsened rapidly. In another primary metastatic patient (bones, lung), NE differentiation of the metastatic sites was confirmed and SSTR-targeted therapy with lanreotide was successfully administered for several months. Further octreotide scans and 68Ga-DOTATOC PET/CT were performed during follow-up in this patient to assess therapy response. Three other patients with unclear findings on conventional radiologic imaging received an octreotide scan to exclude metastatic lesions with NE differentiation.

Fourteen patients (52%) received a mastectomy, while breast conserving surgery was performed in 11 patients (41%). Two patients had no surgical procedure, one because of stage IV disease at the time of diagnosis and one due to her poor general condition (*advanced cardiovascular disease*). Fourteen patients (52%) were treated with chemotherapy (5 patients received anthracyclines, 2 patients were given taxanes, 7 patients had anthracyclines + taxanes) and 24 (90%) with endocrine therapy. Neuroendocrine-specific treatment with somatostatin analogues was administered in two patients, one diagnosed in stage IV and one diagnosed in stage II. The first patient with stage IV disease and metastases of the bone and lung (T3 N0 M1, G2, Ki-67 25%, HR+/HER2−, SSTR 2 + 70%) received endocrine therapy in combination with lanreotide (120 mg s.c. q4w) after 6 doses of paclitaxel weekly 80 mg/m^2 and achieved complete radiological remission with no evidence of disease at the follow-up of 66 months. At least 60 cycles of lanreotide were administered in combination with endocrine therapy until the last documented follow-up. No SSTR-analogue-specific side effects which altered the therapy regimen were reported. The other patient received the somatostatin analogue octreotide (2 × 50 µg s.c. per day) in stage II (T2 N1 M0, G2, Ki-67 5%, HR+/HER2+, SSTR 2+), after standard therapy was considered unsuitable due to the patient's poor general condition (cirrhosis of the liver (Child's C), thrombocytopenia). Octreotide treatment was administered for 3 months, however this patient died 5 months after diagnosis (no details regarding the exact cause of death or further symptoms and side effects available). ▶ **Table 4** shows the systemic treatment of study patients according to tumor stage and receptor status.

Survival analysis

Follow-up data were available for 26 out of 27 patients. The median follow-up was 63 months (range: 11–170 months). Nine patients died during follow-up and five of 22 initially non-metastatic and R0 operated patients were diagnosed with recurrence (local recurrence and/or distant metastasis). The mean overall survival



▶ **Fig. 4** Kaplan–Meier survival curves of BC-NE patients.

▶ **Table 3** Neuroendocrine-specific immunochemistry findings.

Marker/receptor	n (%)
Total n (%)	27 (100)
Chromogranin A	
▪ > 50 % of tumor cells positive	13 (48)
▪ 1–50 of tumor cells positive	4 (15)
▪ Negative	10 (37)
Synaptophysin positivity	
▪ > 50 % of tumor cells positive	25 (93)
▪ 1–50 of tumor cells positive	2 (7)
▪ Negative	0 (0)
CgA and Syn in > 50 % of tumor cells positive	
▪ Yes	12 (44)
▪ No	15 (56)
SSTR 2A	
▪ Negative	12 (44)
▪ Score 1	2 (7)
▪ Score 2	7 (26)
▪ Score 3	3 (11)
▪ Not evaluated	3 (11)

NE: neuroendocrine, CgA: chromogranine A, Syn: synaptophysin, SSTR 2A: somatostatin receptor type 2A. Numbers in parentheses are percentages and do not add to 100 in some instances owing to rounding.

(OS) was 111 months (95 % CI: 82–140 months), the mean DFS was 124 months (95 % CI: 90–157 months). The 5-year OS rate was 70 % (▶ **Fig. 4**).

For comparison, results from other studies published are summarized in ▶ **Table 5**. Only studies published after 2003 and including at least 20 patients with NEN were considered.

Discussion

Although neuroendocrine differentiation in BC is a long-known phenomenon, first described in 1963 [6], it was not until 2003 that NEN of the breast was defined by the WHO as a distinct subtype. Despite significant advances in the research and treatment of early and metastatic breast cancer over the last decades [11–15], the exact prevalence, clinical behaviour and effective therapy standards for this subset of BC have not been well established so far, possibly due to its low incidence and discrepant definitions.

All patients eligible for our analysis were diagnosed with a NEN of the breast according to WHO 2003 criteria (Syn and/or CgA > 50 %). Poorly differentiated large or small cell neuroendocrine carcinoma and well differentiated neuroendocrine tumors (NET, G1) were excluded from this study (▶ **Table 1**). Since the definition of NEN of the breast has changed twice in the last two decades, the majority of cases described in our study would be currently defined as BC-NE (WHO 2012) and thus, in line with the latest NEN classification 2019, not be classified as a true NEN of the breast (▶ **Table 1**). However, diffuse neuroendocrine differentiation (Syn and/or CgA > 50 %) in BC has been shown to be associated with certain specific clinical features, and several published studies on NEN of the breast report on these tumors as well (▶ **Table 5**). In particular, the question whether neuroendocrine differentiation in BC might have a diagnostic or therapeutic significance has not yet been sufficiently answered.

Here we report on a series of 27 cases of BC-NE and present their clinicopathological characteristics, survival analysis as well as NE-specific diagnostic and therapeutic aspects and compare it with other published studies on NEN of the breast.

▶ **Table 4** Systemic treatment of study patients according to tumor stage and receptor status.

PT	Age	TNM	G	ER	PR	HER2	SSTR 2A score (%)	CT	ET	SSTR therapy
1	61	T1 N0 M0	2	80%	80%	pos.*	2 (60)	No	AI	No
2	46	T4 N1 M1**	2	80%	40%	neg.	1 (< 10)	7 × E q1w	Ful	No
3	73	T2 N0 M0	2	80%	30%	neg.	0	No	AI	No
4	74	T2 N1 M0	2	40%	15%	neg.	0	3 × Pac q1w	AI	No
5	84	T2 N0 M0	2	90%	90%	neg.	2 (60)	No	AI	No
6	62	T3 N0 M1	2	80%	90%	neg.	3 (90)	6 × Pac q1w	AI	Lanreotide 120 mg q4w
7	53	T2 N1 M0	3	80%	0	neg.	1 (< 10)	3 × FEC – 3 × DOC	AI	No
8	72	Tx Nx M0**	2	90%	90%	neg.	2 (70)	No	AI	No
9	51	T1 N0 M0	2	50%	80%	neg.	0	No	AI	No
10	50	T2 N0 M0	2	80%	90%	neg.	3 (90)	6 × FEC q3w	Tam	No
11	42	T2 N3 M0	2	90%	90%	neg.	0	3 × A – 3 × C – 3 × Pac q2w	Tam + GnRH	No
12	38	T2 N0 M0	3	0	0	neg.	0	6 × FEC q3w	No	No
13	53	T2 N3 M1	2	0	0	neg.	n. d.	4 × EC – 4 × DOC	No	No
14	81	T4 Nx M0	2	90%	60%	neg.	3 (90)	No	AI	No
15	80	T2 N3 M0	2	80%	10%	neg.	0	no	AI	No
16	70	T1 N0 M0	2	80%	80%	neg.	0	No	Tam	No
17	56	T2 N0 M0	2	80%	80%	neg.	2 (60)	4 × EC q3w	Tam-AI	No
18	48	T1 N0 M0	2	90%	90%	neg.	n. d.	6 × FEC q3w	Tam	No
19	62	T2 N1 M0	2	80%	20%	neg.	0	3 ×;FEC – 3 × DOC q3w	AI	No
20	84	T2 N0 M0	3	90%	0	neg.	0	No	AI	No
21	72	T1 N0 M0	2	80%	80%	neg.	2 (30)	No	Tam-AI	No
22	56	T1 N1 M0	2	90%	90%	neg.	0	3 × FEC – 3 × DOC q3w	Tam-AI	No
23	51	T2 N1 M0	2	80%	30%	neg.	0	3 × FEC – 3 × DOC q3w	Tam/AI	No
24	60	T1 N0 M0	2	90%	90%	neg.	n. d.	No	Tam/AI	No
25	81	T2 N0 M0	2	50%	< 10%	neg.	0	No	Tam	No
26	56	T2 N0 M0	2	100%	10%	neg.	2 (30)	3 × FEC – 3 × DOC q3w	Tam/AI	No
27	69	T2 N1 M0	2	90%	90%	pos.*	2 (70)	No	No	Octreotide 50 µg 2/d

PT: patient, G: grading, ER: estrogen receptor, PR: progesterone receptor, SSTR 2A: somatostatin receptor type 2A, CT: chemotherapy, ET: endocrine therapy, AI: aromatase inhibitors, Ful: fulvestrant, E: epirubicin, Pac: paclitaxel, F: fluorouracil, C: cyclophosphamide, DOC: docetaxel, A: doxorubicin, d: day, n. d.: not done, q1w: weekly, q2w: every two weeks, q3w every three weeks, * no anti-HER2 therapy administered (PT 1 diagnosed in 2002, PT 27 not-suitable due to cirrhosis of the liver), ** no primary surgery performed (PT 2: stage IV with malignant bone marrow infiltration, PT 8: not suitable due to advanced cardiovascular disease).

Since some patients were identified through clinical records review and others through retrospective staining of neuroendocrine markers, we can only report on the actual prevalence in the collective of 465 patients. With 21 cases identified by a systematic morphological and immunohistochemical re-evaluation, we established a BC-NE prevalence to be 4.5%, which is in line with the 2–5% estimated by the WHO [16]. However, the prevalence of neuroendocrine differentiation in the published studies varies from less than 0.1% [17] to over 20% [18] (▶ **Table 5**). This is due to the variable diagnostic criteria on the one hand and the NEN identification process used in published trials on the other. Analyses that implement the 50% threshold for Syn or CgA according to the WHO 2003 definition generally report lower a NEN prevalence comparing to those meeting WHO 2012 criteria without a threshold and/or using further neuroendocrine markers such as NSE or CD56 for NEN diagnosis [18–21] (▶ **Table 5**). Moreover, trials that identify NEN cases via a review of clinical records or databases report a generally lower and probably underestimated prevalence compared to those which performed a systematic re-evaluation of histology slides from BC patients, since neuroendocrine markers are not routinely used in BC diagnosis [17, 22–24].

The median age at initial diagnosis in our cohort was 61 years, which is in accordance with the median age at diagnosis of breast cancer of no special type without neuroendocrine differentiation (BC-NST) [25]. No differences between NEN of the breast and BC-NST in terms of age at diagnosis have been reported in other

▶ **Table 5** Prevalence, definitions, and clinical characteristics in important studies published on NEN of the breast*.

Study	No. of patients	NEN definition	NEN identification process	Prevalence	Age (range)	Morphology/ initial histology n (%)	IHC staining/ IHC subtype n (%)	Grading n (%)	Tumor size n (%)	N status n (%)	Outcome
Makretsov et al. 2004 [11]	65	Positivity of single NE marker (NSE, CgA or Syn), without threshold	Systematic histological re-evaluation of 334 surgical specimens from 1974–1995	19.5 %	n. r.	n. r.	n. r.	n. r.	n.r	n. r.	No prognostic significance of CgA or Syn expression
	10	> 50 % positivity of single NE marker (NSE, CgA or < Syn)		3 %		IDC (NST) 5 (50) IDC/ILC 2 (20) IDC/MUC 2 (20) MUC 1 (10)	HR+/HER2− 7 (70) HR+/HER2+ 1 (10) HR−/HER2+ 0 (0) TNBC 2 (20)	G1 2(20) G2 7(70) G3 1(10)			n. r.
van Krimpen et al. 2004 [12]	40	Positivity of single NE marker (Syn and/or CgA), no treshold	Histological re-evaluation of 317 surgical specimens from 1983–1990	12,6	n. r.	n. r.	n. r.	n. r.	n. r.	n. r.	No prognostic significance of NE differentiation
Righi et al. 2010 [13]	89	WHO 2003	n.r	n. r.	Median 67 (43–92)	Solid cohesive 35 (39)	ER+ (83) HER2+ (0)	G1 (26) G2 (54) G3 (20)	T1 (62) T2 (31) T3–4 (7)	N0 (71) N+ (29)	n. r.
					Median 68 (54–84)	Alveolar 10 (11)	ER+ (55) HER2+ 0	G1 0 (0) G2 5 (50) G3 5 (50)	T1 (45) T2 (44) T3–4 (1)	N0 (72) N+ (28)	
					Median 62 (39–88)	Small cell 11 (12)	ER+ (67) HER2+ 0	G1 (0) G2 (18) G3 (82)	T1 (17) T2 (83)	N0 (40) N+ (60)	
					Median 71 (27–89)	Solid papillary 20 (22)	ER+ (100) HER2+ 0	G1 (45) G2 (45) G3 (10)	T1 (47) T2 (41) T3–4 (12)	N0 (53) N+ (47)	
					Median 66 (44–87)	Cellular mucinous 13 (15)	ER+ (92) HER2+ 0	G1 (31) G2 (69) G3 (0)	T1 (50) T2 (20) T3–4 (30)	N0 (75) N+ (25)	

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▶ **Table 5** Prevalence, definitions, and clinical characteristics in important studies published on NEN of the breast*. *(Continued)*

Study	No. of patients	NEN definition	NEN identification process	Prevalence	Age (range)	Morphology/ initial histology n (%)	IHC staining/ IHC subtype n (%)	Grading n (%)	Tumor size n (%)	N status n (%)	Outcome
Wei et al. 2010 [14]	74	WHO 2003[1]	Review of clinical records	n. r.	Mean 61 (28–72) Median 63	Solid NE carcinoma Atypical carcinoid Large cell NE carcinoma	ER+ 70 (95) ER− 3 (4) Unknown 1 (1) ER+ 59 (80) ER− 14 (19) Unknown 1 (1) HER2+ 2 (3) HER2− 67 (91) Unknown 5 (6)	G1 2 (3) G2 57 (77) G3 15 (20)	T1 33 (45) T2 31 (42) T3 4 (5) T4 6 (8)	N0 41 (57) N1 31 (42) Unknown 2 (3)	Significantly worse clinical outcome than IDC NST LRFS ($p = 0.001$), DRFS ($p < 0.0001$), and OS ($p = 0.002$)
Riccardi et al. 2011 [15]	22	WHO 2003	Review of clinical records	n. r.	Median 63 (38–74)	n. r.	ER+ 18 (82) ER− 4 (18) PR+ 12 (54) PR− 10 (45) HER2 n. r.	n. r.	n. r.	n. r.	n. r.
Marton et al. 2012 [16]	31	WHO 2003	Review of clinical records; 3058 BC cases diagnosed 2001–2005	1,1 %	61.7 (44–86)	n. r.	ER+ 27 (87) ER− 4 (13) PR+ 23 (74) PR− 8 (26) HER2+ 1 (3) HER2− 30 (97)	G1 7 (23) G2 19 (61) G3 5 (16)	T1 12 (39) T2 18 (58) T3 1 (3) T4 0 (0)	N0 16 (52) N+ 15 (48)	Median follow-up 58.7 months (2–144), disease relapse in 25.8 %, median time to relapse 34.3 months (14.5–54.1)
Rovera et al. 2013 [17]	96	WHO 2012	Review of clinical records, 2829 BC cases diagnosed 1992–2013	3.2 %	Median 70 (40–94)	Solid type 38 (62) MUC 14 (23) Microinvasive 6 (10) LCNEC 2 (3) SCNEC 1 (2)	ER+ (90) PR+ (75) HER2+ 0	G1 (34) G2 (64) G3 (2)	T1 35 (60) T2 20 (34) T3 1 (2) T4 2 (3)	N0 36 (77) N+ 11 (33)	Median follow-up 65 months (range 2–242); 10-year OS 87 %[1]
	61	WHO 2003				n. r.	n. r.	n. r.	n. r.	n. r.	

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▶ **Table 5** Prevalence, definitions, and clinical characteristics in important studies published on NEN of the breast*. *(Continued)*

Study	No. of patients	NEN definition	NEN identification process	Prevalence	Age (range)	Morphology/ initial histology n (%)	IHC staining/ IHC subtype n (%)	Grading n (%)	Tumor size n (%)	N status n (%)	Outcome
Zhang et al. 2013 [18]	107	WHO 2003[1]	Review of clinical records, IHC confirmation	n. r.	Median 64 (25–95)	n. r.	ER+ 101 (94) ER− 6 (6) PR+ 91 (85) PR− 16 (15) HER2+ 3 (3) HER2− 104 (97)		T1 48 (45) T2 54 (50) T3 5 (5) T4 0 (0)	N0 81 (76) N+ 26 (24)	Median follow-up 27 months (3–134); OS 85.1 vs. 92.4 % (NST) (p = 0.030) LRFS NEC (7.5 %) vs. NST (2.8 %) (p = 0.043) DRFS NEC (5 %) vs. NST (8.3 %) (p = 0.061)
Zhu et al. 2013 [19]	22	WHO 2003	Review of clinical records, 7542 BC cases diagnosed 2004–2010	0.29 %	Mean 52.5 (29–77)	n. r.	ER+ 20 (91) ER− 2 (9) PR+ 21 (95) PR− 1 (5) HER2+ 5 (23) HER2− 17 (77)	n. r.	n. r.	n. r.	Mean follow-up 64.5 months (4–89), 95 % of patients disease-free
Cloyd et al. 2014 [20]	284	WHO 2012	Review of SEER database (BC cases diagnosed between 2003 and 2010)	n. r.	n. r.	Well differentiated 148 (52.1) Small cell 73 (25.7) CA with NE features 42 (14.8) Large cell 14 (4.9) Carcinoid 7 (2.5)	ER+ 132 (46.5 %) PR+ 101 (35.6 %) HER2 n. r.	G1 28 (10) G2 56 (20) G3 127 (45) Unknown 73 (26)	T1 87 (31) T2 99 (35) T3–4 51 (18) Unknown 47 (16)	N0 145 (51) N+ 103 (36) Unknown 36 (13)	SCNEC: worse DSS (OR 6.46, 95 % CI: 0.88–47.68, p = 0.07) and OS (1.97, 95 % CI: 0.47–8.22, p = 0.36) compared to other neuroendocrine tumors of the breast
Kwon et al. 2014 [21]	32 59	WHO 2003 WHO 2012	Histological re-evaluation of 1428 surgical specimens from 2012	2.2 % 4.1 %	Median 49	IDC 54 (91.5) MUC 3 (5.1) Micropapillary 2 (3.4)	ER+ 55 (93) ER− 4 (7) PR+ 49 (83) PR− 10 (17) HER2+ 5 (8.5) HER2− 54 (91.5)	G1 8 (14) G2 19 (32) G3 32 (54)	T1 24 (41) T2 32 (54) T3 3 (5) T4 0 (0)	N0 24 (41) N+ 35 (59)	NE differentiation associated with impaired OS (p = 0.004) and DFS (p < 0.001)[3] No difference between focal and diffuse NE differentiation (OS, p = 0.986; DFS, p = 0.861), follow-up 56 months (1–122)

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▶ **Table 5** Prevalence, definitions, and clinical characteristics in important studies published on NEN of the breast*. *(Continued)*

Study	No. of patients	NEN definition	NEN identification process	Prevalence	Age (range)	Morphology/ initial histology n (%)	IHC staining/ IHC subtype n (%)	Grading n (%)	Tumor size n (%)	N status n (%)	Outcome
Park et al. 2013 [22]	87	WHO 2003	Review of clinical records, 12 945 BC cases diagnosed 1984–2011	1 %	Mean 63 (28–89)	IDC 60 (69) IDC/MUC 17 (19.5) IDC/ILC 8 (9.2) Unknown 2 (2.3)	ER+ 86 (99) ER− 1 (1) PR+ 67 (77) PR− 19 (22) Unknown 1 (1) HER2+ 2 (2) HER2− 82 (94) Unknown 3 (3)	G1 8 (9) G2 67 (77) G3 10 (11) Unknown 2 (2)	n. r.	N0 44 (50) N+ 39 (45) Unknown 4 (5)	n. r.
Wang et al. 2014 [23]	142	WHO 2003	Review of SEER database (BC cases diagnosed between 2003 and 2009)	< 0.1 %	Mean 64 (26–99)	n. r.	ER+ 77 (54) ER− 37 (26) Unknown 28 (20) PR+ 53 (37) PR− 59 (42) Unknown 30 (21) HER2+ n. r.	G1 17 (12) G2 30 (21) G3 60 (42) Unknown 35 (25)		N0 52 (37) N+ 40 (28) Unknown 50 (35)	Impaired prognosis compared to BC-NST Median OS 26 months (12–48) 5-year OS 53.6 % (95 % CI: 42.2–63.7) NE differentiation (pos. vs. neg.) DSS 1.80 (95 % CI: 1.36–2.37), p < 0.0001, OS 1.84 (95 % CI: 1.50–2.26), p < 0.0001[3]
Bogina et al. 2016 [24]	84	WHO 2003[1]	Histological re-evaluation of 1232 surgical specimens from 2000–2012	6.8 %		NST 58 (69) ILC 5 (6) MUC 6 (7) Solid papillary 15 (18)	ER+/HER2− (Ki-67 < 14) 34 (41) ER+/HER2− (Ki-67 ≥ 14) 43 (51) ER+/HER2+ 4 (5) ER−/HER2+ 1 (1) TNBC 2 (2)	G1 3 (5) G2 41 (71) G3 14 (24)	T1 51 (61) T2 20 (24) T3–4 13 (15)	N0 38 (30) N+ 31 (37) Unknown 15 (18)	Worse DFS compared to BC-NST, no difference in CSS NE differentiation (pos. vs. neg.) DFS 3.12 (95 % CI: 1.30–7.69), p = 0.011[3]
	128	WHO 2012[1]		10.4 %		NST 95 (74) ILC 5 (4) MUC 7 (6) Solid papillary 21 (16)	ER+/HER2− (Ki-67 < 14) 47 (37) ER+/HER2− (Ki-67 ≥ 14) 65 (51) ER+/HER2+ 9 (7) ER−/HER2+ 3 (2) TNBC 4 (3)	G1 6 (7) G2 65 (68) G3 24 (25)	T1 77 (60) T2 36 (28) T3–4 15 (12)	N0 64 (50) N+ 42 (33) Unknown 22 (17)	

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▶ **Table 5** Prevalence, definitions, and clinical characteristics in important studies published on NEN of the breast*. *(Continued)*

Study	No. of patients	NEN definition	NEN identification process	Prevalence	Age (range)	Morphology/ initial histology n (%)	IHC staining/ IHC subtype n (%)	Grading n (%)	Tumor size n (%)	N status n (%)	Outcome
Roininen et al. 2017 [25]	43	WHO 2003	Review of clinical records, 12 945 BC cases diagnosed 2007–2015	n. r.	Median 66	n. r.	ER+ 41 (96) ER− 1 (2) Missing 1 (2) PR+ 37 (86) PR− 4 (9) Missing 2 (5) HER2+ 2 (5) HER2− 40 (93) Missing 1 (2)	n. r.	T1 29 (67) T2 11 (26) T3 2 (5) T4 1 (2)	N0 24 (56) N+ 17 (39) Missing 2 (5)	Worse DFS (p = 0.024) and OS (p = 0.0028) No difference in DDF, BCSS Mean follow-up of NEN 35.4 months (95 % CI: 23.5–47.2 months)
Kelten Talu et al. 2018 [26]	36	WHO 2003[1]	Review of clinical records and IHC confirmation, BC cases 2007–2016	n. r.	Median 69.5, mean 67.4 (40–88)	IDC + NE differentiation 28 (78) Solid NEC 2 (5) IDC/MUC 2 (5) MUC 2 (5) IDC/ILC 1 (3) Solid papillary carcinoma 1 (3)	HR+/HER2− 33 (91.6) HR+/HER2+ 2 (5.6) TNBC 1 (2.7)	G1 0 (0) G2 31 (86) G3 5 (14)	T1 13/36 (36) ≥ T2 21/36 (58)	n. r.	No conclusions
Lavigne et al. 2018 [27]	47	WHO 2003	Review of clinical records	n. r.	Median 67, mean 69 (33–91)	NST 37 (79) ILC 2 (4) Solid papillary carcinoma 5 (11) MUC 3 (6)	ER+ 47 (100) ER− 0 (0) PR+ 36 (77) PR− 10 (21) Unknown 1 (2) HER2+ 1 (2) HER2− 46 (98)	G1 3 (6) G2 29 (62) G315 (32)	T1 28 (60) T2 16 (34) T3 2 (4) T4 1 (2)	N0 22 (47) N+ 18 (38) Unknown 7 (15)	Impaired DFS, no difference in OS
Our study	27	WHO 2003[1]	Histological re-evaluation of 465 surgical specimens from 2002–2006, review of clinical records 2007–2013	4,5 %	Median 61 (28–84)	NST 16 (59) ILC 1 (4) NST/ILC 1 (4) MUC 4 (15) NET 5 (18)	HR+/HER2− 23 (85) HR+/HER2+ 2 (7) HR−/HER2+ 0 (0) TNBC 2 (7)	G1 0 (0) G2 21 (78) G3 6 (22)	T1 7 (26) T2 16 (60) T3–4 3 (11) Unknown 1 (4)	N0 15 (56) N+ 10 (37) Unknown 2 (7)	Median follow-up 63 months (11–170), 5-year OS 70 %

* Studies and case series published after 2003 with at least 20 patients have been listed. Numbers in parentheses are percentages and do not add to 100 in some instances owing to rounding. Abbreviations: NEN: neuroendocrine neoplasia, SCNEC: small cell neuroendocrine carcinoma, LCNEC: large cell neuroendocrine carcinoma, CSS: cancer specific survival, CgA/B: chromogranin A/B, Syn: synaptophysin, NSE: neuron-specific enolase, DRFS: distant recurrence-free survival, LRFS: local recurrence-free survival, DSS: disease-specific survival, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma; MUC: mucinous, NST: no special type, BC-NST: breast cancer of no special type, DFS: disease free survival, OS: overall survival, n. r.: not reported. [1] No SCNEC and/or LCNEC included, [2] no cases with > 50 % positivity, [3] multivariate analysis

case series [19, 26, 27]. However, several trials with large cohorts reported NEN of the breast patients to be significantly older than BC-NST patients [17, 28 – 30], These discrepancies may also be due to nonuniform diagnostic criteria used in published series: most of the studies meeting WHO 2003 criteria report NEN of the breast patients being significantly older than BC-NST patients [17, 28 – 30] (▶ **Table 5**).

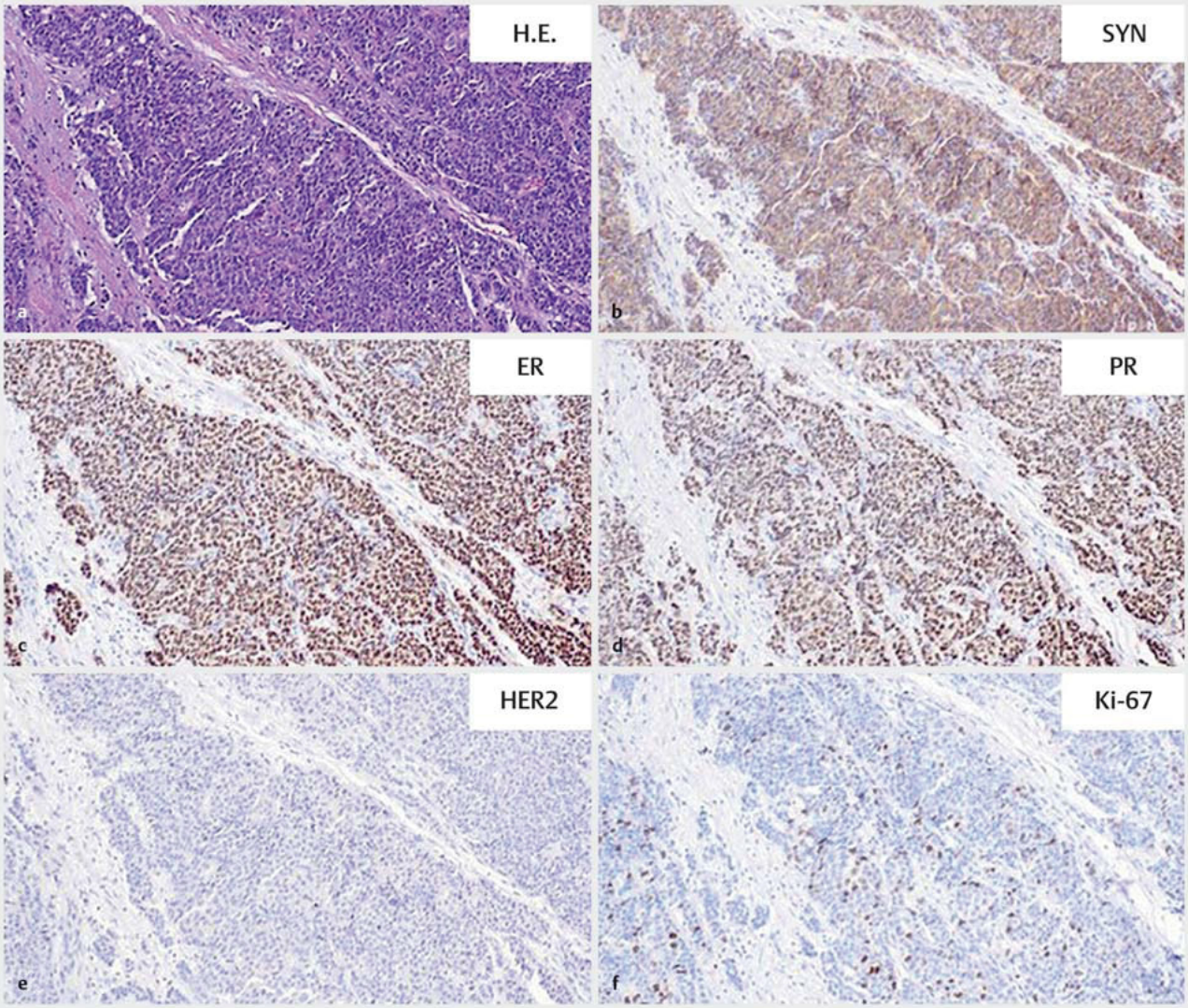
The majority (60 %) of patients in our cohort were diagnosed with ≥ T2 tumors, and 37 % of our analysed patients had lymph node metastases. This observation, i.e., NEN of the breast being diagnosed at a higher TNM stage than BC-NST, has also been reported by others. Wang et al. in their study of 142 NEN of the breast patients showed that those tumors were significantly larger, had higher stage disease and were significantly often node-positive compared to control cohorts with BC-NST [17]. In the study by Cloyd et al. of 284 patients, NEN of the breast was associated with relatively more advanced disease than BC-NST [31]. In their trial of 128 cases, Bogina et al. reported that NEN patients presented with larger tumors than BC-NST patients but no difference regarding node status was observed [19]. In contrast, some, mostly small series, reported similar TNM stages at diagnosis between BC with and without neuroendocrine differentiation [18, 26 – 28]. The proposed rationale for this phenomenon in NEN of other locations is their low grading and therefore slow growth, resulting in a lack of early symptoms. However, the association with higher TNM stages has been also reported in NEN cohorts with high rates of poorly differentiated tumors [17, 31].

Similar to previous studies, the majority of patients (85 %) in our analysis presented with ER-positive HER2-negative tumors (▶ **Fig. 5**) [17, 22, 27, 32]. Previously, neuroendocrine differentiation has been shown to be significantly associated with positive HR-status [19, 26, 30] and negative HER2-status [28, 29]. Most tumors in our analysis were G2 tumors (78 %) and Ki-67 was higher than 30 % in 11 of 27 patients (41 %). Similarly, NEN patients in other series were shown to have G2 tumors significantly more often than patients with BC-NST [19, 28], whereas some studies reported NEN being of a significantly higher histologic grade [17] and others found no association between neuroendocrine differentiation and grading [26, 27]. These discrepancies may be due to inconsistent NEN cohorts, since particular subtypes of NEN are associated with certain pathological features. In the trial by Cloyd et al., 45 % NEN patients presented with poorly differentiated or undifferentiated tumors. However, 26 % of NEN analyzed were SCNEC, well known for poor differentiation [33] and this entity has been excluded from several studies on NEN of the breast, including our analysis. In contrast, studies that analyzed primarily mucinous NEN demonstrated that the majority of these patients had well differentiated tumors [34, 35]. As mentioned above, due to different diagnostic criteria and the fact that specific subtypes within NEN have not been reported in most analyses (e.g., solid NEC vs. well differentiated NET vs. BC-NE vs. SCNEC/LCNEC), the comparison and interpretation of published data is difficult (▶ **Tables 1** and **5**).

The question whether neuroendocrine differentiation affects the prognosis of BC patients remains a very much debated issue. The 5-year OS rate of 70 % in our cohort of patients with BC-NE is lower than the OS in patients with BC-NST [25]. Although some smaller studies reported similar [18, 20, 21, 36] or even better [32, 37, 38] outcomes for NEN compared to BC-NST patients, the majority of published large series demonstrated an impaired prognosis for NEN [17, 19, 26 – 30] and most of these studies do not include any SCNEC cases, well known for having a very poor outcome [19, 26 – 29]. The association with poor clinical outcome was also present in multivariate analysis after adjusting for pathological stage [17, 26], histological grade, and ER and HER2 status [19, 26], showing that neuroendocrine differentiation is an independent prognostic factor in BC.

Expression of somatostatin receptor (SSTR) in NEN of the breast, similarly to NEN of other sites, is a long-known phenomenon [39], potentially allowing SSTR-targeted tumor imaging and treatment, even though it is not restricted to this subset of BC [40]. Among them, SSTR 2A is a subtype most commonly expressed in BC [41] and able to mediate the antiproliferative effect of somatostatin analogues (SSA) in the strongest manner [42]. However, the SSTR 2A positivity rate in BC-NE has, to the best of our knowledge, only been analyzed in one study so far [43]. This recently published retrospective analysis of 31 NEN cases reported a SSTR 2A positivity rate of 71 % [43]. In our series, SSTR 2A was evaluated in 24 patients and 12 of them (50 %) were SSTR 2A-positive. Based on this, five patients received SSTR-based imaging (octreoscan or ^{68}Ga-DOTATOC PET/CT) to confirm or exclude metastatic disease at the time of diagnosis or to evaluate therapy response over the course of disease. It is possible that the number of patients receiving SSTR-based imaging would have been much higher if neuroendocrine differentiation had been identified at diagnosis and not, as was the case in the majority of our BC-NE patients, retrospectively.

Beyond these specific diagnostic aspects, SSTR 2A can potentially be targeted with SSA such as octreotide or lanreotide. These substances, which have been a mainstay of antisecretory treatment in functional NEN for a long time, were also shown to have antiproliferative activity and to be associated with a clinical benefit in some NEN patients [44]. In NEN of other sites, which is much more common, this therapy is mainly being considered in well differentiated NET (G1/2, Ki-67 < 10 %) [45]. Current recommendations for BC-NE therapy are based on general guidelines for breast cancer, and poorly differentiated SCNEC (▶ **Table 1**) is the only entity with specific recommendations (i.e., platinum/etoposide-based chemotherapy similar to small cell lung cancer). However, only a few case reports on the treatment of BC patients with this regimen have been published so far [46, 47]. Since this rare subtype of NEN of the breast known to have a very poor outcome has been excluded from our analysis, all patients in our study were treated with a standard anthracycline-taxane (AT)-based chemotherapy. In our series, two SSTR-positive BC-NE patients received SSA in combination with endocrine therapy and one of these patients, initially diagnosed at stage IV with metastasis to lung and bones, achieved complete remission showing no evidence of disease on radiological and SSTR-based imaging 66 months after the first diagnosis. This patient exhibited strong SSTR 2A-expressing BC-NE G2 with a Ki-67 of 25 % and not a typical well differentiated NET. Indeed, SSA therapy has been evaluated in BC-NST in the past and showed response rates of up to 40 % in a metastatic setting in phase I–II trials [48]. However, a phase III study comparing



▶ **Fig. 5** Expression of receptors and proliferative activity in breast carcinoma with neuroendocrine differentiation. **a** Hematoxylin and eosin (H. E.) staining, demonstrating a solid growth pattern, complete lack of tubular architecture and a cytology of tumor cells highly suggestive of a neuroendocrine phenotype. **b** Strong expression of the pan-neuroendocrine marker synaptophysin (SYN) in all tumor cells. **c** Strong nuclear expression of the estrogen receptor (ER) in > 90 % of tumor cells resulting in an ER score of 12 (scale 0–12). **d** Strong nuclear expression of the progesterone receptor (PR) in > 90 % of tumor cells resulting in an ER score of 12 (scale 0–12). **e** Complete lack of HER2 expression corresponding to a score of 0 (scale 0–3). **f** Analysis of Ki-67 protein expression reveals a proliferative activity of approximately 15 %.

endocrine therapy with or without octreotide in primary ER-positive BC did not show a benefit of SSA treatment in this setting [49]. Nonetheless, none of these studies evaluated the SSTR status of tumor tissue prior to SSA-based therapy. Here we demonstrate that SSA therapy in SSTR 2A-positive BC-NE can be offered as an individual treatment option to selected patients, e.g., as combination therapy in a palliative setting or in the case of contraindications to the standard treatment. Since neuroendocrine differentiation has been shown to be associated with impaired outcomes in several retrospective trials, further studies are needed to identify the most appropriate treatment strategy for this BC subtype.

Conclusion

Invasive breast cancer with neuroendocrine differentiation represents mostly HR-positive and HER2-negative disease and the diagnosis is made at a higher TNM stage than for BC-NST. Neuroendocrine differentiation in BC has been shown to be associated with impaired prognosis in several retrospective trials. However, the clinical impact of NE features in BC is still a very much debated issue, since the diagnostic criteria of this entity differ in published studies, making an estimation of clinical behavior difficult. Current recommendations for BC-NE therapy are based on general guidelines for breast cancer. Nevertheless, a significant number of these cancers express SSTR 2A receptors, allowing SSTR-based

imaging and potentially SSTR-targeted therapy in selected cases. Moreover, platinum/etoposide-based chemotherapy may be an alternative to the standard AT-based treatment in poorly differentiated SCNEC of the breast.

Declarations Section

Ethics approval and consent to participate: The study was approved by the Ethical Committee of the Heinrich Heine University of Duesseldorf.

Consent to publish: This manuscript does not contain any details, images, or videos that might lead to the identification of any individual patient.

Availability of data and materials section: The data that support the findings of this study are available from the authors on reasonable request and with the permission of Tanja Fehm.

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Authors' contribution: NK performed the data analysis and drafted the manuscript. RR collected the data and helped to draft the manuscript. SO, KL helped to perform the IHC experiments. MA, and SB performed the IHC experiments, the morphological evaluation and helped to draft the manuscript, IE perform the IHC experiments and help to draft the manuscript, CM helped to draft the manuscript. MN designed and coordinated the study, TF designed the study, made substantial contribution to interpretation of the data and reviewed the manuscript. MBP, ER, SM, JH, TK, BJ were involved, in interpretation of the data, drafting of the manuscript or revising it. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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