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Habilitationsschrift

Primäre und sekundäre Resistenzmechanismen der EGFR Blockade

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Einführung

Die Familie der Her-Rezeptoren und Entwicklung von Substanzen zur Inhibition in der Krebstherapie

Zelluläres Wachstum wird durch Signalübertragung an Wachstumsrezeptoren reguliert. Wachstumsfaktoren und deren Rezeptoren sind für diesen Prozeß essentiell und vermitteln Signale zwischen der Zellumgebung und dem Zellkern. Die „epidermal growth factor receptor“ Familie umfaßt vier in enger Beziehung stehender Rezeptoren: EGFR1 bzw. Her-1, Her-2, Her-3 und Her-4. Es handelt sich um transmembranäre Glykoproteine mit einer extrazellulären Bindungsdomäne und einer intrazellulären Tyrosinkinasedomäne über die Wachstumsregulation stattfindet.(1) Kommt es zur Dysregulation im Sinne einer Überexpression oder dauerhaften Aktivierung der Rezeptoren wird eine Tumorentstehung begünstigt.(2) Her-1 und Her-4 gelten als autonome Rezeptoren, d.h. nach Ligandenbindung findet eine Dimerisierung und nachfolgende Aktivierung der Signalkaskade statt. Im Unterschied dazu fehlen dem Her-2 Rezeptor die eigenen Liganden und Her-3 fehlt die Tyrosinkinasedomäne, so dass diese Rezeptoren auf eine Heterodimerisierung zur Aktivierung angewiesen sind. Liganden an den drei Rezeptoren sind unter anderen TGF-alpha, Amphiregulin, Neuregulin, Epiregulin, Betacellulin und der epidermal growth factor (EGF).(3) Eine gesteigerte Aktivierung des Rezeptorsignalings resultiert in Proliferation von Endothelzellen, Neubildung von Blutgefäßen, Inhibition der Apoptose und Metastasierung. Mechanistisch ist meist die Überexpression des Rezeptors, die zur einer gesteigerten Aktivierung der Signalkaskade führt. Daneben kommen aber ebenso aktivierende Mutationen und die Überexpression von Liganden vor. Die Phosphorylierung der Tyrosinkinase des EGFR führt zu einer Aktivierung eines komplexen Netzwerks von Signalkaskaden. Das Ras-Raf-MAPK System ist einer der Hauptwege durch die die Signalübertragung zum Zellkern erfolgt.(4)

Aufgrund der nachgewiesenen Rolle des EGFR in der Tumorentstehung und Progression maligner Erkrankungen wurden viele Substanzen entwickelt, die intra- oder extrazellulär zu einer Blockade der Signalübertragung führen. Eine der ersten lizensierten und in der Therapie von Krebserkrankungen bewährten Substanzen ist Cetuximab, ein chimerer IgG1 Antikörper gegen den EGF-Rezeptor. Cetuximab ist ein 152-kDa Molekül bestehend aus vier Polypetidketten, zwei identischen Schwerketten (γ) und zwei identischen Leichtketten (κ) zusammengesetzt aus 449 und 214 Aminosäuren, die durch kovalente und nicht kovalente Bindungen gehalten werden.

Die Bindung zwischen Cetuximab und dem EGFR ist gekennzeichnet durch eine höhere Affinität (Kd = 0.1–0.2 nM) als die der endogenen Liganden EGF und TGF-alpha, wodurch eine Aktivierung des Rezeptors verhindert wird.(5)
Neben Cetuximab wurde Panitumumab, ein voll humanisierter monoklonaler Antikörper gegen den EGFR entwickelt, der ebenfalls extrazellulär bindet. Im Unterschied zu den Antikörpern, die extrazellulär binden, wurden eine Reihe von Tyrosinkinaseinhibitoren (TKIs) entwickelt, die eine Aktivierung des intrazellulären Anteils des Rezeptors vermindern. Zu diesen Substanzen gehören Gefetinib, Erlotinib, Afatinib oder auch Osimertinib. Erstaunlich ist die unterschiedliche Wirkung der Antikörper und der TKIs in verschiedenen Krankheitsentitäten. Die Tyrosinkinaseinhibitoren haben einen festen Stellwert in der Behandlung von Lungenkarzinomen beim Vorliegen einer EGFR Mutation, während die Antikörper eine vergleichbare Wirkung in dieser Tumorentität nicht belegen konnten. Dagegen zeigt sich weder bei kolorektalen Karzinomen noch bei Kopf-Hals Karzinomen, bei denen die monoklonalen Antikörper ein fester Bestandteil der Therapie sind, eine Wirkung der Tyrosinkinaseinhibitoren.(6)
Die BOND Studie (Bowel Oncology with Cetuximab Antibody) führte erstmals zur Zulassung von Cetuximab bei Patienten, die an einem metastasierten kolorektalen Karzinom (CRC) erkrankt waren. In der Studie wurde Cetuximab allein oder in Kombination mit Irinotecan bei 329 Patienten mit metastasierten kolorektalen Karzinom mit dem Ergebnis einer Ansprechrate von 22,9% im Kombinationsarm und einem Ansprechen von 10,8% bei alleiniger Antikörper Gabe untersucht. Weitere Studien folgten mit unterschiedlichen Kombinationspartnern, die die Wirksamkeit der Substanzklasse beim CRC belegen konnte.(7)

Wirkmechanismen von Cetuximab und anderen EGFR-Inhibitoren

Wie in Abbildung 1 dargestellt konkurrieren Cetuximab und die Liganden des EGFR um die Bindungsstelle am Rezeptor.(8) Kommt es zur Bindung von Cetuximab bleibt eine Phosphorylierung der Tyrosinkinase und eine Aktivierung der Signalkaskade aus. Darüber hinaus erfolgt die Internalisierung des Rezeptors und nachfolgender Abbau, woraus eine Abnahme der Expression resultiert.(9) Ein dritter Mechanismus, der zur Wachstumsinhibition und zytotoxischen Reaktion führt, beruht auf der Eigenschaft, dass Cetuximab als chimerer Antikörper mit einem humanen und einem murinen Anteil synthetisiert wurde. Der murine Anteil wird vom körpereigenen Immunsystem als fremd

erkannt, was eine Aktivierung des Immunsystems zur Folge hat. Die antibody dependent cellular cytotoxicity (ADCC) führt zur Einwanderung von NK- und zytotoxischen T-Zellen, die in einer Lyse der Antikörper tragenden Zellen resultiert. Ein Effekt der im Rahmen einer Tumortherapie durchaus gewünscht ist. Die Eigenschaft eine ADCC zu induzieren, unterscheidet Cetuximab von Panitumumab, dem voll humanisierten Antikörper.(10)

Durch die verminderte Phosphorylierung des EGFR entwickelt sich ein Zellzyklus Arrest. G1-Phase Progression und Eintritt in die S-Phase sind abhängig von Cyclin-abhängigen Kinasen (CDK), insbesondere CDK4, CDK6 und CDK2. Cetuximab bewirkt eine Abnahme der CDKs bei gleichzeitig vermehrten Auftreten von Apoptose.(11) Eine weitere antiproliferative Wirkung wird durch die verminderte Ausschüttung von angiogenen Wachstumsfaktoren erreicht, was zu einer Abnahme der Dichte der kleinen Gefäße führt.(12)

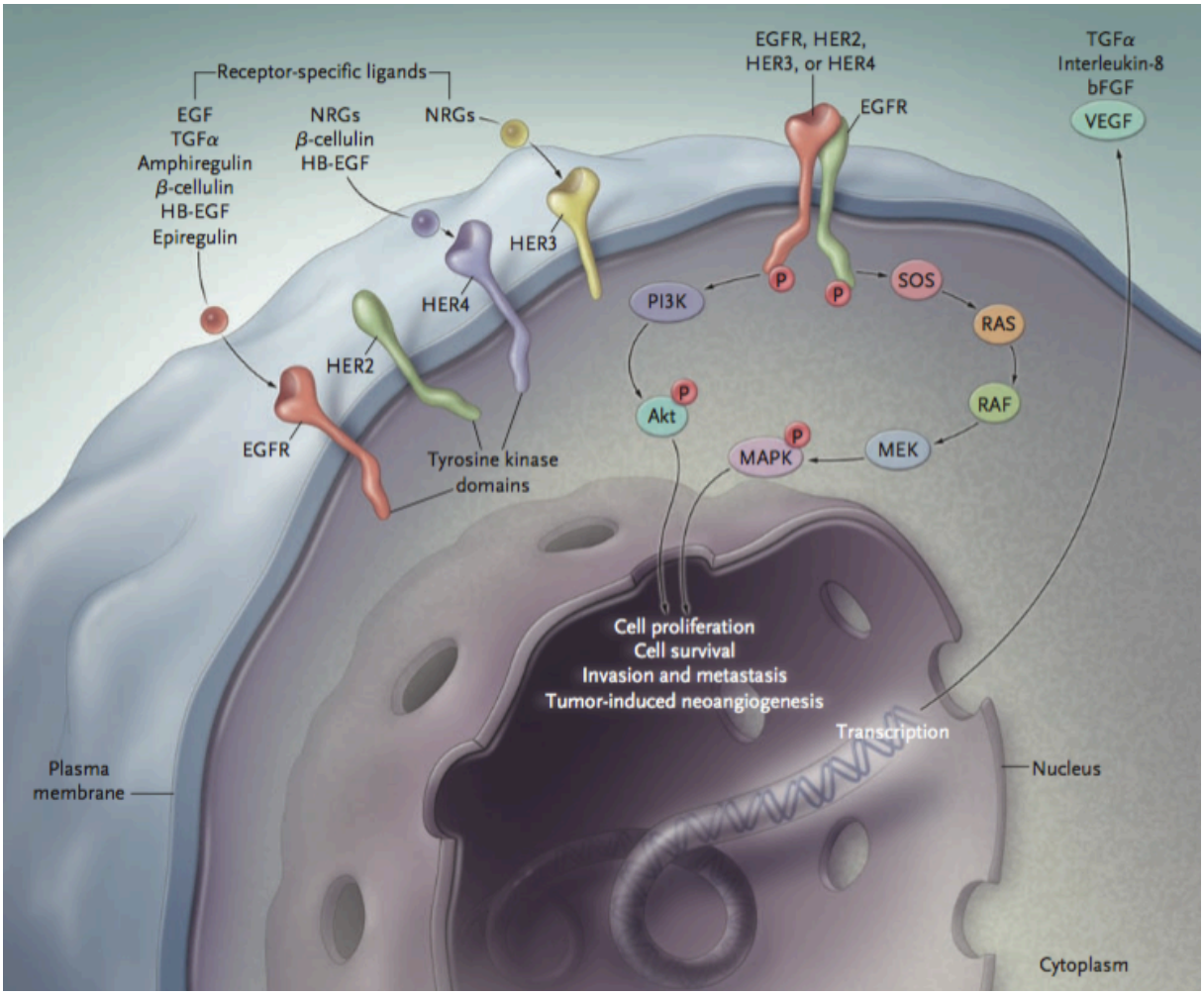

Abbildung 1 Signaltransduktion am EGFR Reproduced with permission from Ciardello, F et al N Engl J Med 2008; EGFR Antagonists in Cancer Treatment 358:1160-1174), Copyright Massachusetts Medical Society.

Kopf-Hals Karzinome – Inzidenz und Entwicklung der zielgerichteten Therapie

Kopf-Hals Karzinome umfassen eine heterogene Gruppe von Tumoren, die aufgrund der anatomischen engen Lagebeziehung zusammengefaßt werden. Darüber hinaus handelt es sich bei über 90% der Tumore um Plattenepithelkarzinome. In Deutschland erkranken knapp 20.000 Menschen an Kopf-Hals Karzinomen pro Jahr.(13) Trotz Präventionsprogrammen, verbesserter Operations- und Bestrahlungstechniken erleiden ca. 50% der Patienten ein Tumorrezidiv. Im Rezidiv ist die palliative Systemtherapie der therapeutische Standard. Platinbasierte Therapieregime führten zu den höchsten Responseraten und diese lassen sich durch Kombination mit Taxanen oder 5-FU steigern.(14, 15) Bis 2008 konnte mit der Systemtherapie zwar eine Symptomlinderung belegt werden, eine Verbesserung des Gesamtüberlebens gelang aber erst durch den Einsatz von Cetuximab.(16) EGFR-Antikörper in der Entität der Kopf-Hals Karzinome zu evaluieren erschien sinnvoll, da sich in Screeningstudien gezeigt hatte, dass 90-100% der Plattenepithelkarzinome des Kopf-Hals Bereichs die molekulare Zielstruktur exprimieren.(17) Der Antikörper wurde zunächst in der BONNER Studie in Kombination mit Bestrahlung in der kurativen Situation untersucht. Dabei zeigte sich ein signifikant längeres progressionsfreies (24,4 vs 14,9 Monate) und Gesamtüberleben (49 vs 29,3 Monate) gegenüber der alleinigen Strahlentherapie. In der EXTREME Studie erfolgte parallel die Evaluation in Kombination mit der Chemotherapie in der palliativen Situation. Die Kombination von Platin, 5-FU und Cetuximab führte zu einem signifikant längeren Überleben und wurde dadurch zum neuen Therapiestandard in der palliativen Situation.(16) In der SPECTRUM Studie wurde das Studiendesign der EXTREME Studie mit Panitumumab wiederholt. Trotz vergleichbarer Wirkprinzipien und vergleichbarer Patientenpopulationen zeigte sich in dieser Studie kein Vorteil in Hinblick auf ein verlängertes Überleben. Inwieweit die ADCC diesen Unterschied bedingt oder aber das Fehlen der Erhaltungstherapie nach Abschluß der Chemotherapie, ist bis heute unklar, weil es einen direkten Vergleich der beiden Antikörper im Rahmen einer Studie nicht gab. Cetuximab war damit die erste und bis 2017 die einzige zugelassene zielgerichtete Therapie in der Behandlung von Kopf-Hals Plattenepithelkarzinomen. Eine Reihe weitere EGFR Antikörper wurden in der Vergangenheit entwickelt. Beispiele dafür sind u.a. ICR62, ABX-EGF, Matuzumab, Nimotuzumab und zuletzt eine glykosilierten Form von Cetuximab, das CetuGEX. Diese Substanzen wurden unterschiedlich weit entwickelt, konnten aber keine

verbesserte Wirkung bzw ein längeres Überleben gegenüber dem zuerst eingeführten Cetuximab belegen. (18)
Bedauerlicherweise profitieren nicht alle Patienten von einer EGFR Blockade, so dass eine Patientenselektion anhand von tumorbiologischen oder genetischen Markern des Patienten wünschenswert erscheint.

Korrelation zwischen Hauttoxizität und EGFR Blockade

Allen Substanzen, die am EGFR binden ist die Nebenwirkung eines charakteristischen Hautexanthems gemeinsam, das mehr als 50% der Patienten entwickeln. Ein papulo-pustolöses Exanthem, welches prädominant im Gesicht und der oberen Thoraxapertur auftritt. Die Ausprägung des Exanthems variiert zwischen Patienten erheblich. Die pathogenetischen Mechanismen, die zur Entwicklung des Exanthems führen, sind nicht vollständig verstanden. In der Epidermis spielt die Regulation durch den EGFR eine wichtige Rolle hinsichtlich der Entwicklung und Ausdifferenzierung der Haut, Schutz vor UV-induzierten Strahlenschäden sowie Kontrolle der Inflammation und Wundheilung.(19) Medikamentös induzierte Blockade des EGFR führt zur Störung der Proliferation, Differenzierung und Migration von Keratinozyten, was eine papulo-pustolöse Reaktion und Xerosis hervorrufen kann.(20) Histologisch ergibt sich das Bild einer Inflammation mit einer Entzündungsinfiltration in den höher gelegen Schichten der Dermis im Bereich der Hautfollikel. Die Behandlung mit EGFR Inhibitoren führt zur verminderten Phosphorylierung am EGFR und konsekutiv verminderten Expression von MAPK. In basalen Keratinozyten bewirkt dies einen Wachstumsstop und vorzeitige Differenzierung, die normalerweise erst in höheren Schichten der Haut stattfindet. Die Blockade führt dadurch zur Ausschüttung von Chemokinen, die wiederum zur Rekrutierung von Leukozyten führen und letztlich das klinische Bild bedingen.(21)
Interessant ist das beschriebene Phänomen zwischen dem Auftreten der Hauttoxizität und der Wirksamkeit der Therapie. Die meisten Untersuchungen liegen zum kolorektalen Karzinomen vor. In der OPUS Studie wurde ein Zusammenhang zwischen der Intensität des Exanthems und dem Ansprechen auf die Therapie berichtet. Diese bestand aus Cetuximab in Kombination mit einer Chemotherapiekombination bestehend aus Oxaliplatin, 5 FU und Folinsäure (FOLFOX). Lediglich 13% der Patienten, die kein Hautexanthem ausbildeten, zeigten ein Ansprechen. Im Vergleich dazu waren die Ansprechraten 42.2% bei einem Grad 1, 53.2% bei Grad 2, und 66.7% falls Grad 3 oder 4 vorlag.(22) Die Beobachtung wurde

in der CRYSTAL Studie untermauert durch die positive Korrelation zwischen progressionsfreien Überleben und dem Grad der Hauttoxizität.(23) Auch beim Lungenkarzinom, Kopf-Hals Karzinom und Pankreaskarzinom haben sich Hinweise für die beschriebene Korrelation ergeben.(24)

Prognostische und Prädiktive Biomarker zur individualisierten Tumortherapie

Unter einem Biomarker wird ein biologisches Merkmal verstanden, das objektiv gemessen und bewertet werden kann.(25) Biomarker werden in der Behandlung von Krebserkrankungen vor allem eingesetzt, um Aussagen zur Prognose des Patienten zu treffen. Ein Beispiel dafür ist, dass Patienten mit einer TP53 Mutation bei Kopf-Hals Karzinomen im Tumor eine schlechtere Prognose als ohne diese haben. TP53 Mutationsstatus ist demnach ein prognostischer Biomarker. Möchte man eine Aussage über die Wirksamkeit einer Therapie anhand eines biologischen Merkmals treffen, so benötigt man einen prädiktiven Biomarker. Ein Beispiel aus der Krebsmedizin ist das Fusionsprodukt zwischen bcr und abl bei der CML. Durch den Nachweis des bcr-abl Fusionsprodukts ist eine Wirksamkeit von Imatinib hochwahrscheinlich. Umso besser der prädiktive Marker, so besser gelingt die Selektion des Medikaments für den einzelnen Patienten. Prädiktive Biomarker lassen sich nur über das molekulare Verständnis der Erkrankung und die molekularen Mechanismen der eingesetzten Substanz ermitteln.

Zielsetzung

Ziel der experimentellen Arbeiten war und ist es, ein verbessertes molekulares Verständnis der Kopf-Hals Karzinome zu entwickeln, um somit eine Therapieselektion für den individuellen Patienten zu ermöglichen. Vor dem Hintergrund, dass es bis 2017 nur eine einzige zugelassene zielgerichtete Therapie für die Kopf-Hals Karzinome gab, konzentrierten sich die Arbeiten auf die Identifikation prädiktiver Biomarker zum Einsatz von Cetuximab und der Analyse primärer und sekundärer Resistenzmechanismen. Die Arbeiten zur Identifikation von Biomarkern wurde zunächst an Patientenmaterial durchgeführt. Translationale Forschung mit archivierten Tumormaterial birgt eine Reihe von Limitationen:

1. Verfügbarkeit: Ein häufiges Problem ist die eingeschränkte Verfügbarkeit und möglicherweise das fehlendes Einverständnis der Nutzung von Tumormaterial durch den Patienten.

2. Zeitliche Differenz: In der Regel wird Tumorgewebe von Patienten zum Zeitpunkt der Diagnosestellung entnommen. Im Rezidiv, wenn eine medikamentöse Behandlung stattfindet und eine Korrelation tumorbiologischer Eigenschaften zu einer Therapie hergestellt werden sollen, ist das Gewebe des Primärtumors unter Umständen nicht mehr repräsentativ.
3. FFPE fixiertes Material unterliegt einer Degradierung der Nukleinsäuren, so dass eine Vielzahl von Untersuchungen insbesondere von RNA Expressionsstudien an diesem Material nur eingeschränkt umsetzbar sind.
4. Ein Vergleich zwischen unbehandeltem Gewebe und behandeltem Gewebe ist an Patientenmaterial selten möglich.

Aufgrund dieser Limitation wurde die Entwicklung und Etablierung eines geeigneten präklinischen Modells verfolgt, das zum einen die Heterogenität der Kopf-Hals Karzinome abbildet, nah am Ausgangstumor bleibt und eine ausreichende Geweberessource für translationale Forschung darstellt. Patientenabgeleitete Xenograftmodelle eignen sich für diese Fragestellungen, deshalb war die Etablierung und Charakterisierung die Grundlage für weiterführende experimentelle Arbeiten.

Eigene Arbeiten

Polymorphismen des EGFR

Klinghammer K, Knodler M, Schmittel A, Budach V, Keilholz U, Tinhofer I. **Association of epidermal growth factor receptor polymorphism, skin toxicity, and outcome in patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment.** Clin Cancer Res. 2010;16(1):304-10

Rezeptorpolymorphismen können zu unterschiedlich starker Bindung der Liganden und der inhibierenden Antikörper führen. Inwieweit das Phänomen der Hauttoxizität unter EGFR-Blockade von EGFR Polymorphismen abhängt, also dem Genotyp des Patienten, wurde in einer Kohorte von Kopf-Hals Tumorpatienten untersucht, die im Rahmen einer Zweitlinientherapie Cetuximab und Docetaxel erhalten hatten. Die CETAX Studie war eine unverblindete, nicht randomisierte Phase II Studie, die in der deutschen Studiengruppe (AIO) durchgeführt wurde. Von 51 der 84 in der Studie behandelten Patienten wurde Tumormaterial untersucht. Hauttoxizitäten wurden entsprechend der Common Toxicity Criteria einheitlich bewertet und für jeden Patienten während der Behandlung dokumentiert. 21 der 51 Patienten (41%) entwickelten eine Hauttoxizität > Grad1 unter Cetuximab/Docetaxel. Der Genotyp von zwei unterschiedlichen EGFR Polymorphismen R521K und ein CA *repeat* (CA-SSR) Polymorphismus im Intron 1 wurden mit dem Grad der Hauttoxizität korreliert. Dabei zeigte sich, dass der G/G Genotyp des R521K Polymorphismus signifikant mit einem höheren Grad an Hauttoxizität korrelierte und mit einem verminderten Risiko einer Tumorprogression assoziiert war (Hazard ratio, 0.55; 95% confidence interval, 0.27-1.08; P = 0.08). Ein Zusammenhang von dem CA *repeat* Polymorphismus und dem Auftreten von Hauttoxizität oder Überleben der Patienten zeigte sich nicht. In nachfolgenden Arbeiten anderer Arbeitsgruppen konnte gezeigt werden, dass die Bindungsaffinität durch den R521K SNP beeinflußt wird. Eine prospektive Evaluation, inwieweit die genetische Variante als prädiktiver Biomarker verwendet werden kann, ist bisher nicht erfolgt.

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Clinical Cancer Research

Cancer Therapy: Clinical

Association of Epidermal Growth Factor Receptor Polymorphism, Skin Toxicity, and Outcome in Patients with Squamous Cell Carcinoma of the Head and Neck Receiving Cetuximab-Docetaxel Treatment

Konrad Klinghammer[1], Maren Knödler[1], Alexander Schmittel[1], Volker Budach[2], Ulrich Keilholz[1], and Ingeborg Tinhofer[2]

Abstract

Purpose: Cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR), has shown clinical efficacy in squamous cell carcinoma of the head and neck with prolonged progression-free (PFS) and overall survival (OS). In this study, we analyzed whether cetuximab-induced skin rash was correlated with distinct polymorphisms within the *EGFR* gene known to modulate EGFR expression, ligand binding, or signaling activity.

Experimental Design: Fifty-one patients enrolled in a single-arm phase II multicenter study for second-line treatment of recurrent or metastatic squamous cell carcinoma of the head and neck with cetuximab/docetaxel were genotyped for two genetic variations in the *EGFR* gene, a point substitution G→A in exon 13 resulting in an amino acid substitution in position 521 (EGFR-R521K) and a CA repeat (CA-SSR) polymorphism in intron 1. Association between genotypes and incidence/grade of skin rash was determined by Fisher's exact test. The predictive value of genotypes for PFS and OS was determined using the log-rank test.

Results: Overall, 21 patients (41%) developed skin rash with grade >1 within 6 weeks of treatment. The common EGFR-R521K genotype (G/G) was significantly associated with increased skin toxicity ($P = 0.024$) and showed a trend toward reduced risk of tumor progression (hazard ratio, 0.55; 95% confidence interval, 0.27-1.08; $P = 0.08$), whereas no correlation of the EGFR-R521K genotype with OS could be observed ($P = 0.20$). No significant interaction between CA-SSR polymorphism and skin toxicity, PFS, or OS could be detected.

Conclusions: Our study revealed an influence of the EGFR-R521K genotype on skin toxicity and suggested its relation to clinical activity of cetuximab/docetaxel treatment. *Clin Cancer Res; 16(1); 304–10. ©2010 AACR.*

Epidermal growth factor receptor (EGFR) and its ligands play a fundamental role in signaling transduction pathways involved in DNA repair, tumor cell survival, proliferation, and metastasis. In addition, EGFR overexpression in tumor tissue has frequently been associated with poor clinical outcome. In squamous cell carcinoma of the head and neck (SCCHN), overexpression of EGFR has been shown (1, 2) and associated with decreased response to therapy (3, 4) and reduced disease-free and overall survival (OS; ref. 4, 5). Because of its prevalence and its crucial role in the pathogenesis, targeting EGFR has become a rational approach for treatment of SCCHN. Indeed, the combination of cetuximab with radiotherapy (6) or platinum-containing chemotherapy regimens (7) has already shown significant improvement of treatment outcome. In search for biomarkers allowing prospective identification of patients with significant benefit of EGFR targeting therapy, the role of gene amplification, protein expression levels, and activating mutations in EGFR itself or in key molecules downstream its specific signaling pathway have been evaluated by numerous studies. They revealed significant association between these biomarkers and the efficacy of treatment in lung (8–11) and colorectal cancer (12, 13). However, activating mutations in EGFR and downstream signaling molecules are rather rare in SCCHN and, together with EGFR expression levels, seem not to influence treatment efficacy in SCCHN (14, 15). However, the latter study was the first to reveal a significant association between the development of skin rash of at least

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Translational Relevance

Anti–epidermal growth factor receptor (EGFR) therapy with cetuximab for treatment of squamous cell carcinoma of the head and neck has shown promising results even in patients with recurrent/metastatic disease who experienced disease progression on platinum therapy. As potential mechanisms that may reduce sensitivity to cetuximab treatment, EGFR amplification as well as activating K-ras mutations have been identified in colorectal cancer, but such genetic aberrations are rarely detected in squamous cell carcinoma of the head and neck. Here, we show for the first time that a germline polymorphism of *EGFR* (EGFR-R521K), which has been associated with EGFR ligand binding and its mitogenic activity, could predict the occurrence of cetuximab-related skin toxicity, the most frequent adverse side effect of cetuximab treatment that has also been associated with its clinical efficacy. Indeed, patients with the G/G genotype of EGFR-R521K who significantly developed skin rash more often showed a trend to prolonged progression-free survival on cetuximab/docetaxel treatment. Larger clinical trials are needed to confirm and validate our preliminary findings.

grade 2 and OS of SCCHN patients treated with erlotinib (15), an observation subsequently made also in other tumor entities (16). The molecular basis for this association is still unresolved; however, two genetic variations in the *EGFR* gene affecting either EGFR expression levels (17) or ligand binding affinity (18) have been proposed as possible cause. The first one, a highly polymorphic microsatellite sequence in intron 1 (9-23 CA simple sequence repeat here called CA-SSR) has been shown to influence the expression levels of EGFR in a way that the longer the CA repeat, the lower the EGFR expression is (19) and the less effective EGFR targeting is in SCCHN cell line models (17). The second one is a single nucleotide polymorphism (G→A) in exon 13 (called EGFR-R521K in this study) that leads to an amino acid exchange from arginine to lysine with the EGFR-R521K variant (A genotype) showing less affinity to EGF and transforming growth factor-α and less mitogenic activity than the common G genotype (18). In the present study, we evaluated the potential of these two genetic variations to predict for the development of skin rash and treatment outcome in patients with advanced SCCHN receiving cetuximab/docetaxel treatment.

Materials and Methods

Patients and treatment. Eighty-four patients with histologically confirmed recurrent or initially metastatic SCCHN were enrolled in a phase II multicentric clinical trial for treatment with cetuximab/docetaxel. Further eligibility criteria were tumor relapse after cisplatin-containing chemoradiotherapy or after platinum-containing first-line chemotherapy; no intermittent anticancer treatment since platinum failure; Eastern Cooperative Oncology Group performance status 0 to 1; adequate bone marrow, liver, and renal function; and signed written informed consent. Eligible patients received a maximum of six cycles of 35 mg/m^2 of docetaxel administered on day 1, 8 and 15, repeated on day 29 in the absence of disease progression or severe toxicity. Cetuximab was administered at an initial dose of 400 mg/m^2 followed by subsequent weekly doses of 250 mg/m^2 until disease progression or severe toxicity. Tumor assessment was done after every 8 wk. Evaluation of treatment response was done according to Response Evaluation Criteria in Solid Tumors. Skin toxicity was recorded according to National Cancer Institute common toxicity criteria, version 3. Highest recorded grade of skin rash during the first cycle of treatment was used for evaluation. All patients for whom skin reactions during treatment had been documented and from whom tumor biopsy material was available (n = 51 from 5 of 10 participating clinical study centers) were included in the accompanying translational research study presented here.

DNA extraction and EGFR polymorphism analysis. Genomic DNA was extracted from paraffin-embedded tissue samples using QIAmp DNA FFPE Tissue kit (Qiagen) according to the manufacturer's instruction. DNA content and quality was determined using the Nanodrop 1000 spectrophotometer. The EGFR-R521K polymorphism (rs11543848) was analyzed by RFLP-PCR as described previously (20). Briefly, for amplification of the designated region in exon 13 of the EGFR gene, the forward primer (5′-TGC TGT GAC CCA CTC TGT CT-3′) and reverse primer (5′-CCA GAA GGT TGC ACT TGT CC-3′) were used. Each PCR was done in a total volume of 50 μL containing 150 ng genomic DNA, 5 μL 10× PCR buffer including 1.5 mmol/L $MgCl_2$ (Roche), 200 nmol/L of each primer, 1 μL Nucleotide Mix (Roche) equivalent to 200 nmol/L of each nucleotide, and 2.5 units of Taq DNA Polymerase (Roche). After initial denaturation at 94°C for 3 min, the reaction was carried out at 94°C denaturation for 30 s, 58.6°C annealing for 30 s, and 72°C elongation for 30 s for a total of 30 cycles. Twenty microliters of PCR product was digested overnight with BstNI restriction enzyme (New England Biolabs) at 60°C. Fragments were separated on a 4% agarose gel (NuSieve, Lonza). Results were visualized by staining gels for 45 min with Sybr Green (Sigma). For validation of RFLP-PCR results, EGFR-R521K genotypes from four samples were confirmed by sequence analysis of the PCR product.

Genotyping of EGFR CA-SSR (rs11568315) was done by PCR and consecutive double-strand sequencing. Primers to amplify the CA repeat region were designed using the Primer3 program. The forward primer 5′-GGG CTC ACA GCA AAC TTC TC-3′) and reverse primer 5′-AAG CCA GAC TCG CTC ATG TT-3′ were used.

Statistical analysis. The influence of EGFR polymorphisms on the incidence/severity of skin toxicity, as the

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primary hypothesis of this study, and on the disease control rate [DCR; partial remission (PR) and stable disease (SD)] was assessed using Fisher's exact test. The level of significance was set at $P < 0.05$. The P values from the additional statistical analyses of associations between EGFR polymorphisms and progression-free survival (PFS) or OS were regarded as exploratory because the cohort size had not the statistical power for such type of analysis. PFS was calculated from the date of initiation of cetuximab/docetaxel therapy to the date of disease progression, the date of death if it occurred before documented progression, or the date of last contact. OS was calculated from the date of treatment initiation to the date of death or the date of last contact. Comparison of PFS and OS from different genotype groups was done using the Kaplan-Meier method and significance was determined using the log-rank test. Statistical analyses were carried out using the StatView software (version 5.0.1, SAS Institute, Inc.).

Results

Relationship between EGFR genotypes and patient characteristics. The relative distribution of EGFR genotypes and their association with patient characteristics are given in Table 1. For EGFR-R521K, the relative frequency of A and G genotypes was comparable with that reported by the HapMap consortium for a Caucasian reference cohort: G/G (n = 24, 47%) was the most common genotype and 27 patients showed the variant form, of which 4 (7%) were homozygous (A/A) and 23 (45%) were heterozygous (G/A). No significant association of the G or A genotype with sex, age, tumor localization, or initial tumor stage was observed (Table 1).

The number of CA repeats within the microsatellite region of EGFR intron 1 comprised between 14 and 21. As reported for healthy controls as well as SCCHN patients, we observed a predominance of 16 CA repeats (21). In detail, the allelic distribution was as follows: 33%, 16-CA; 17.6%, 15-CA; 16.7%, 17-CA; 12.7%, 20-CA; 9.8%, 18-CA; 4.9%, 19-CA; 3.9%, 21-CA; and 0.9%, 14-CA. We used a length of ≤16 CA repeats in the shorter allele as cutoff for the definition of two genotype groups because this cutoff has been shown to distinguish between EGFR expression levels (19) and between the response of non–small cell lung carcinoma (NSCLC) patients to gefitinib treatment (22, 23). No significant association of CA-SSR with sex, age, tumor site, or stage was observed (Table 1).

EGFR genotypes and therapy-induced skin toxicity. Evaluation of EGFR polymorphisms that have been shown to influence the expression of EGFR (19) or its affinity to EGF/transforming growth factor-α (18) was made and associated with the development of skin toxicity during the first cycle of treatment. Patients were stratified in two groups according to skin toxicity of grade 0 to 1 or grade >1 because by using this cutoff, a significant association between CA-SSR and skin rash has recently been identified in lung cancer patients treated with gefitinib (24). In our cohort, a correlation between the allelic length of the CA-SSR and the occurrence of skin rash was not observed (P = 0.99; Table 2). In contrast, the A genotype (A/A or G/A) of EGFR-R521K was significantly associated with a lower incidence of skin rash grade >1 compared with the G/G genotype (P = 0.024; Table 2).

Table 1. Baseline patient characteristics according to EGFR polymorphisms

Factor	Total	R521K A/A or G/A genotype	R521K G/G genotype	*P*	CA-SSR >16	CA-SS ≤ R16	*P*
Sex (*n*)							
Female	11	7	4		7	4	
Male	40	20	20	0.50	18	22	0.32
Age							
Median (SD)	60 (8)	59 (7)	64 (9)	0.40	63 (8)	59 (8)	0.23
Tumor site (*n*)							
Oral cavity	11	4	7		5	8	
Oropharynx	16	8	8		4	12	
Hypopharynx	14	7	7		5	9	
Larynx	1	1	0		0	1	
Other	3	2	1	0.71	1	2	0.93
Tumor stage at initial diagnosis (*n*)							
I	8	5	3		6	2	
II	2	1	1		2	0	
III	2	1	1		1	1	
IV a	27	13	14		9	18	
IV b	3	0	3		2	1	
IV c	4	3	1		3	1	
Not available	5	4	1	0.40	2	3	0.20

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Table 2. Association of EGFR polymorphisms with skin toxicity

Factor	Total	R521K A/A or G/A genotype	R521K G/G genotype	*P*	CA-SSR > 16	CA-SSR ≤ 16	*P*
Skin rash (*n*)							
Grade 0/1	30 (14/16)	20	10		15	15	
Grade 2/3	21 (20/1)	7	14	0.024	10	11	0.99

Skin toxicity and EGFR-R521K as predictors for response, PFS, and OS. Because the efficacy of cetuximab, either as monotherapy in metastatic colorectal cancer (25) or combined with radiotherapy in SCCHN (6) or chemotherapy in NSCLC (FLEX study ref. 26),[3] has been positively associated with incidence/grade of skin toxicity, we evaluated whether the occurrence/severity of skin toxicity was associated with response, PFS, or OS in our study cohort. Response rate for all 51 patients was 12% and the DCR (DCR = PR + SD) was 55%. Median PFS and OS were 4.1 and 7.5 months, respectively. There was a trend for an association between skin toxicity and treatment efficacy: in patients without skin rash (grade 0), PR and DCR were 7% and 28%, 13% and 63% for those with a skin toxicity of grade 1, and 14% and 67% for those with a skin toxicity of grade >1, respectively (with $P = 0.07$ for DCR). Although we observed a trend toward longer PFS and OS for those patients experiencing any kind of rash, the grade of skin toxicity itself had no influence on PFS or OS (Fig. 1).

The major advantage of a molecular biomarker other than skin rash for the prediction of the efficacy of EGFR targeting would be that such molecular marker could be determined before initiation of cetuximab treatment and, thus, could be included in the algorithm of treatment decision. Since observing a significant association of the EGFR-R521K genotype with skin rash (Table 2), we finally asked whether EGFR-R521K was also associated with outcome of SCCHN patients treated with cetuximab/docetaxel. In the group of patients with the A genotype of the EGFR-R521K (A/A or G/A), PR and DCR was 7% and 44% compared with 17% and 67% in patients with the G genotype of EGFR-R521K ($P = 0.16$ for DCR). Furthermore, patients with the A genotype of the EGFR-R521K showed a trend to shorter PFS (hazard ratio, 0.55; 95% confidence interval, 0.28-1.08; log-rank: $P = 0.08$; Fig. 2A) but the EGFR-R521K genotype had no influence on OS (hazard ratio, 0.68; 95% confidence interval, 0.36-1.29; log-rank: $P = 0.20$; Fig. 2B), probably due to the small cohort size. As for skin rash, we did not observe an association of the CA-SSR genotype (≤16 CA repeats) with DCR ($P = 0.49$), PFS (log-rank: $P = 0.36$), or OS (log-rank: $P = 0.69$).

[3] U. Gatzemeier, J. von Pawel, I. Vynnchenko, et al., presented at the 2008 Chicago Multidisciplinary Symposium in Thoracic Oncology.

Discussion

In the present study, we found that the occurrence of skin rash under EGFR antibody treatment of patients with advanced head and neck cancer was associated with the EGFR-R521K genotype. Although the underlying molecular mechanisms remain unclear, an attenuated antibody binding to the variant form of EGFR might be the cause for less skin rash and shorter PFS and OS in patients with A genotype. Indeed, structural analysis of the molecular interaction between the Fab fragment of cetuximab and the extracellular domain of EGFR revealed that amino acid exchanges at critical interaction sites dramatically influenced binding affinity not only of EGF itself but also of cetuximab (27). However, because the effect of an arginine-to-lysine exchange at codon 521 had not been tested in this previous study, the interaction of the EGFR-R521K genotype with cetuximab binding affinity remains unresolved.

Thus far, EGFR-R521K has not been associated with the occurrence of skin rash in EGFR antibody regimens. Three recent studies failed to observe a correlation between EGFR-R521K and skin toxicity in NSCLC patients treated with gefitinib (24, 28) or erlotinib (29). These differences in results could be explained by the different EGFR-targeting agents used in their and our study: tyrosine kinase inhibitors such as gefitinib or erlotinib bind to the intracellular tyrosine kinase domain of the EGFR and should therefore not interfere with the extracellular ligand binding domain affected by the EGFR-R521K; thus, an influence of a polymorphism would only be related to the binding of the ligand or cetuximab to the receptor and not to the inhibitory function of the tyrosine kinase inhibitor. A second explanation might be a difference based on the ethnic background of patients (Asian versus Caucasian), which has been shown to significantly affect the activity of EGFR-targeting therapy for genetically ill-defined reasons (30). Of course, differing results about the association of EGFR-R521K with skin toxicity may also arise from differences in treatment regimens due to potential confounding effects by docetaxel. However, an influence of docetaxel in our cohort seems very unlikely because skin toxicity is a very rare event in treatment with docetaxel and, if observed at all, manifests itself rather as erythema at hands and feet and not as the clinically typical EGFR inhibition–related rash (7).

Increased affinity between EGF or transforming growth factor-α and the EGFR of R521K common genotype leading to an enhanced transcription of *fos, myc*, and *jun* has initially

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been shown by Moriai and coworkers (18). Subsequently, several studies identified the EGFR-R521K genotype as independent prognostic factor. Zhang et al. (31) found that colorectal cancer patients with G/G genotype of EGFR-R521K had a higher risk of local tumor recurrence than patients with G/A or A/A genotype. In accordance with this observation, Wang et al. (32) defined EGFR-R521K as a key determinant factor for tumor recurrence of stage II/III colorectal cancer after curative surgery. Given that the prognostic value of EGFR-R521K results from its interference with the affinity of EGFR ligand binding and signaling activity, patients with the unfavorable G/G genotype and thus increased EGFR pathway activity should mostly benefit from EGFR-targeting therapy, at least if further genetic alterations such as activating *raf* or *ras* mutations rendering ligand-mediated EGFR activation irrelevant did not occur. Indeed, even in the small patient cohort of our study, we observed a trend to prolonged PFS in patients carrying the G genotype (Fig. 2), which would support our hypothesis of preferential activity of EGFR targeting in this patient cohort. The small sample size of our study clearly limits our conclusions on the association of EGFR-R521K with clinical efficacy of cetuximab. However, support for our hypothesis of increased activity of cetuximab in patients carrying the G genotype of EGFR-R521K comes from a recent report on patients with metastatic colorectal cancer receiving cetuximab monotherapy who had a better survival if they had the G/G or G/A compared with A/A genotype of EGFR-R521K (33).

Based on our results, it is tempting to speculate that a predictive molecular biomarker with the characteristics of EGFR-R521K would have several advantages compared with the observation of skin rash after treatment initiation. First, given an association of EGFR-R521K with decreased cetuximab binding, it might identify patients for whom the dose of cetuximab potentially would have to be intensified. Second, the results from our study and those from previous reports testing the predictive value of EGFR-R521K for the efficacy of cetuximab or tyrosine kinase inhibitors (24, 28, 29), respectively, suggest that EGFR-R521K might be a drug-specific, but not class-specific, marker. It can thus be hypothesized that EGFR-R521K has the potential to identify patients who are poor responders to cetuximab and for whom treatment with tyrosine kinase inhibitors would be potentially more successful. Of course, both hypotheses, EGFR-R521K as a biomarker for the adaptation of dose and/or selection of the class of drug, will have to be tested in further preclinical and clinical studies.

In contrast to the observed association of EGFR-R521K with skin toxicity, we did not find such an association for CA-SSR despite *in vitro* evidence that this specific polymorphism interferes with EGFR expression levels of cell lines (19, 34) and their sensitivity to the growth-inhibitory effect of erlotinib (17). The results from the subsequent clinical evaluation of CA-SSR as predictive marker for skin toxicity and response to EGFR targeting therapy were less conclusive: in 84 patients with NSCLC, the analysis of the CA-SSR together with 14 further single nucleotide polymorphisms within the EGFR gene detected a significant association between the number of CA repeats and the response to gefitinib treatment if a cutoff of ≤16 CA repeats was used (22). A second study in a cohort of 58 NSCLC patients could detect such an association only if patients were stratified into two groups defined by having 16 CA repeats or any other number (23). Using a cutoff of ≤18 CA repeats for grouping 52 NSCLC patients, Huang et al. (24) found a significant association of the number of CA-SSR only with skin rash but not with tumor response. Finally, the evaluation of the relationship between CA-SSR and EGFR expression levels in SCCHN could detect an inverse correlation of CA repeat numbers and EGFR expression only in a subgroup of 76 patients who exhibited at least one 16 CA repeat allele (21). An association of CA-SSR genotype with patient survival was not found in either in this patient subgroup or in the total cohort. Although testing each of the proposed cutoffs for definition of two risk groups, a significant association between CA-SSR, skin toxicity, and outcome was not found, which speaks against the clinical potential of this genetic markers.

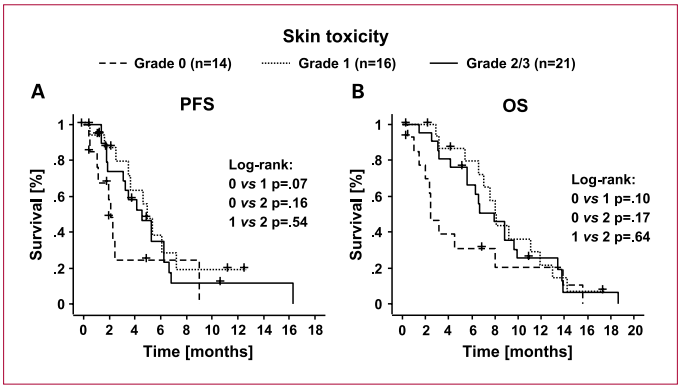

Fig. 1. SCCHN patients with any skin toxicity on docetaxel/cetuximab treatment show a trend for prolonged PFS and OS. Kaplan-Meier curves for PFS (*A*) and OS (*B*) of patients with skin toxicity grade 0, grade 1, or grade >1 are presented. *P* values for comparison of groups using the log-rank test are given.

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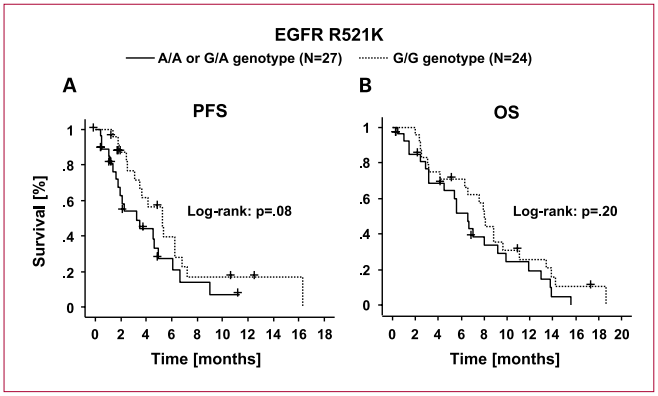

Fig. 2. The EGFR-R521K genotype influenced PFS but not OS of SCCHN patients treated with docetaxel/cetuximab. Kaplan-Meier curves for PFS (*A*) and OS (*B*) of SCCHN patients with the A/A or G/A genotype (*solid line*) or the G/G genotype of the EGFR-R521K (*dotted line*) are presented, and *P* values for comparison of groups using the log-rank test are given.

In conclusion, EGFR-R521K but not CA-SSR polymorphism might be an attractive predictor for the occurrence of cutaneous side effects. Its potential value in predicting efficacy under EGFR-targeting antibody treatment has to be validated in clinical trials including larger patient cohorts.

Disclosure of Potential Conflicts of Interest

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Der Zusammenhang von Amphiregulin und EGFRvIII Expression und Therapieansprechen bei Kopf-Hals Tumorpatienten

Tinhofer I, Klinghammer K, Weichert W, Knodler M, Stenzinger A, Gauler T, et al. **Expression of amphiregulin and EGFRvIII affect outcome of patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment**. Clin Cancer Res. 2011;17(15):5197-204

Um weitere mögliche prädiktive Biomarker für das Ansprechen einer EGFR gerichteten Therapie zu untersuchen, wurde die Expression des EGF-Rezeptors, die Expression des Liganden Amphiregulin und das Vorliegen einer Mutation des EGFR in einem Studienkollektiv von Kopf-Hals Tumorpatienten untersucht. Bei Patienten mit kolorektalen Karzniomen konnte gezeigt werden, dass eine hohe Proteinexpression von Amphiregulin (AREG) mit einem geringeren Überleben assoziiert ist (26) und Patienten mit hoher RNA Expression von Amphiregulin eher auf eine EGFR Blockade ansprechen.(27) Zum Kopf-Hals Karzinom lagen keine Daten vor, so dass die Rolle von AREG untersucht wurde. Ergänzt wurden die Untersuchungen durch PCR basierten Nachweis einer EGFR Mutation. Bei der Variante III des EGFR kommt es zum Verlust von Exon 2-7, woraus ein 150 kDa Protein mit konstitutioneller Aktivierung des Singalwegs resultiert. Eine Assoziation zwischen Ansprechen bzw. dem Überleben der Patienten mit Kopf-Hals Karzinom und einer zielgerichteten Therapie war bislang nicht untersucht. 47 Patientenproben aus der CETAX Studie wurden für diese Analysen untersucht und ausgewertet. Es zeigte sich, dass eine hohe Expression von EGFRvIII oder AREG mit einem geringeren Ansprechen auf die Therapie mit Cetuximab/Docetaxel einherging.

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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

Expression of Amphiregulin and EGFRvIII Affect Outcome of Patients with Squamous Cell Carcinoma of the Head and Neck Receiving Cetuximab–Docetaxel Treatment

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Abstract

Purpose: Constitutive activation of epidermal growth factor receptor (EGFR) as a result of gene amplification, mutation, or overexpression of its ligands has been associated with response to EGFR targeting strategies. The role of these molecular mechanisms for the responsiveness of squamous cell carcinoma of the head and neck (SCCHN) to cetuximab-containing regimens remains unknown.

Experimental Design: Tumor biopsies from 47 patients, enrolled in a single-arm phase II multicenter study for second-line treatment of recurrent or metastatic SCCHN with cetuximab and docetaxel, were analyzed by immunohistochemistry for expression of EGFR, its deletion variant III (EGFRvIII) and its ligand amphiregulin (AREG). The relation between expression levels and disease control rate (DCR) was evaluated by logistic regression. Association between expression levels, progression-free survival (PFS), and overall survival (OS) was determined by Kaplan–Meier analysis, log-rank test, and uni- and multivariate Cox regression analysis.

Results: High expression of EGFR, EGFRvIII, and AREG was detected in 73%, 17%, and 45% of SCCHN cases, respectively. Expression levels of EGFR had no impact on PFS or OS. High expression levels of EGFRvIII were significantly associated with reduced DCR and shortened PFS (HR: 3.3, $P = 0.005$) but not with OS. Patients with high AREG expression in tumor cells had significantly shortened OS (HR: 2.2, $P = 0.002$) and PFS (HR 2.2, $P = 0.019$) compared with patients with low expression score. Multivariate Cox analysis revealed an independent association of AREG and EGFRvIII with PFS but only AREG was an independent prognosticator of OS.

Conclusions: High EGFRvIII and AREG expression levels identify SCCHN patients who are less likely to benefit from combination treatment with cetuximab and docetaxel. *Clin Cancer Res; 17(15); 5197–204. ©2011 AACR.*

Introduction

Epidermal growth factor receptor (EGFR) plays an important role in tumor growth, invasion, and metastasis and is commonly overexpressed in a variety of epithelial malignancies. In squamous cell carcinoma of the head and neck (SCCHN), EGFR expression has been reported in up to 90% of tumors (1, 2), and high expression levels have not only been associated with a more aggressive phenotype but also with decreased responsiveness to radio- or chemotherapy (3, 4) and reduced relapse-free and overall survival (OS) (4–6). Because of its prevalence and crucial role in the pathogenesis, targeting EGFR has thus become a rational approach for treatment of SCCHN. Indeed, combinations of cetuximab with radiotherapy (7, 8) or platinum-containing chemotherapy regimens (9) have already shown significant improvement of treatment outcome. However, for these cetuximab-containing treatment modalities, a local 2-year control rate of 50% (7) and a best overall response rate of 36% (9), respectively, were shown suggesting that a considerably large group of patients will not benefit from cetuximab.

In the search for biomarkers allowing prospective identification of patients with significant benefit of EGFR targeting therapy, the predictive role of EGFR gene

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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amplification, activating mutations, and protein expression levels as well as the expression of EGFR ligands has already been evaluated. These studies revealed that EGFR gene amplification (10) or expression of EGFR (7, 9) did not predict for the response of SCCHN patients to EGFR targeting. EGFR-activating mutations are rather rare in SCCHN (11, 12) and thus, contrary to lung cancer, less likely to contribute to treatment outcome. In contrast, EGFR deletion variant III (EGFRvIII), which has been shown to be expressed in about 45% of SCCHN cases, was shown to be significantly associated with reduced response to cetuximab treatment in SCCHN cell line models (13). Regarding the role of EGFR ligands, high expression levels of amphiregulin (AREG) and epiregulin have been associated with improved disease control on cetuximab monotherapy (14) or combined cetuximab–irinotecan treatment (15) in patients with metastatic colorectal cancer (mCRC). In the SCCHN model, the predictive value of EGFR ligands is less well established and rather conflicting data exist: overexpression of AREG was found to be associated with reduced response to cetuximab or gefitinib in 1 study of SCCHN cell lines (16), whereas 2 other studies again using SCCHN cell lines as a model identified high AREG mRNA and protein expression as predictors for high sensitivity to EGFR targeting (17, 18).

Considering the paucity of information on the role of EGFRvIII and AREG for the response to EGFR targeting in SCCHN patient cohorts, we retrospectively evaluated their role for cetuximab efficacy within a multicenter clinical phase II trial for treatment of recurrent/metastatic SCCHN patients with cetuximab in combination with docetaxel.

Materials and Methods

Patients and treatment

Eighty-four patients with histologically confirmed recurrent or initially metastatic SCCHN were enrolled in a phase II multicentric clinical trial for treatment with cetuximab and docetaxel. Further eligibility criteria were tumor relapse after platinum-containing chemoradiotherapy or after platinum-containing first-line chemotherapy, no intermittent anticancer treatment since platinum-failure, ECOG performance status 0–1, adequate bone marrow, liver, and renal function, and signed written informed consent. Eligible patients received a maximum of 6 cycles of docetaxel (35mg/m^2) administered on days 1, 8, and 15, repeated on day 22 in the absence of disease progression or severe toxicity. Cetuximab was administered at an initial dose of 400 mg/m^2 followed by subsequent weekly doses of 250 mg/m^2 until disease progression or severe toxicity. Tumor assessment was done after every 8 weeks. Evaluation of response according to Response Evaluation Criteria in Solid Tumors was carried out at 3 months while on treatment. Progression-free survival (PFS) was calculated from the date of initiation of cetuximab–docetaxel therapy to the date of disease progression, date of death if it occurred before documented progression, or date of last contact. OS was calculated from the date of treatment initiation to the date of death or the date of last contact. All patients from whom tumor biopsy material was available ($n = 47$ from 5 of 10 participating clinical study centers) were included in the accompanying translational research study presented here.

Immunohistochemistry

Expression analysis of EGFR, EGFRvIII, and AREG was conducted on archival formalin-fixed paraffin-embedded (FFPE) primary tumor biopsy material. For the evaluation of AREG expression, conventional 3-μm sections from the original tissue blocks were used. For the analysis of EGFR and EGFRvIII expression, tissue microarrays (TMA) were generated using a precision instrument (Beecher Instruments). Two tissue cylinders of 1.5-mm diameter were punched from each tumor-bearing donor block, transferred to a TMA recipient block, and serial 3-μm sections were cut. For cases which were not informative on the TMA or for which the biopsy material was too small in size for a TMA, conventional 3-μm sections from the original tissue blocks were used for staining.

EGFR expression was detected by the EGFR pharmDx Kit for Manual Use (Dako), according to the manufacturer's instructions. Immunohistochemical analysis of EGFRvIII was done using mAb L8A4 specific for the junction of the fusion of exons 1 to 8 found in EGFRvIII (ref. 19; kindly provided by Dr. Bigner, Duke University Medical Center, Durham, North Carolina). For specificity control of mAb L8A4, immunostaining was carried out using 3-μm sections from the FFPE glioma cell line U87 stably transfected with an EGFRvIII expression vector or an empty vector (kindly provided by Dr. Furnari, University of California, San Diego). Analysis of AREG expression was conducted using a polyclonal goat anti-AREG antibody raised against the cleaved mature form of AREG (clone AF262; R&D Systems).

After heat-induced antigen retrieval, slides were incubated with the specific primary antibody (mAb L8A4, dilution 1:200; clone AF262, dilution 1:75) at 4°C overnight. The omission of the primary antibody served as negative control. Bound antibody was detected by a Super Sensitive IHC Detection System (BioGenex), according to the manufacturer's protocol. For color development, a Fast Red system (Sigma, Deisenhofen, Germany) was used. The slides were coverslipped after counterstaining.

Evaluation of immunostaining

Immunostainings were independently analyzed by 2 pathologists (W.W. and A.S.) who were blinded to the clinical data. The membrane, cytoplasmic, and nuclear expression levels of EGFR, EGFRvIII, and AREG were scored applying a semiquantitative scoring system considering the staining intensity and area extent. Every tumor was given a score according to the intensity of the staining (no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3) and the extent of stained cells (0% = 0, 1%–10% = 1%, 11%–50% = 2, 51%–80% = 3, and 81%–100% = 4). The final immunohistochemistry (IHC) score

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was determined by multiplying the intensity scores with the extent of positivity scores of stained cells which resulted in a minimum score of 0 and a maximum score of 12. For all 3 markers, the consistency of scoring between the 2 observers was high [kappa scores (ref. 20): EGFR, 0.79; EGFRvIII, 0.83; AREG, 0.86].

Detection of EGFRvIII transcripts by reverse transcription PCR

For independent validation of IHC results, we selected 4 EGFRvIII-positive and 4-negative FFPE samples as well as the FFPE glioma cell line U87 stably transfected with an EGFRvIII expression vector or an empty vector for detection of EGFRvIII transcripts using the protocol established by Yoshimoto and colleagues (21). Briefly, total RNA from 3 to 5 10-μm slices of FFPE tumor tissue was extracted using the RecoverAll Total Nucleic Acid isolation Kit (Applied Biosystems/Ambion), according to the manufacturer's protocol, with the exception that the proteinase K treatment was prolonged to 4 hours to increase RNA yield. RNA was quantified using the NanoPhotometer from Implen. Synthesis of cDNA was carried out in a total volume of 25 μL with 500-ng random hexamers (Roche) and 2 μg of total RNA using the Omniscript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to the supplied protocol. cDNA (2 μL) was used for the PCR reaction. The quality of RNA was checked by PCR detection of the housekeeping gene porphobilinogen deaminase (PBGD; forward primer: 5′- GGC ACA ACC GGG TGG GGC A-3′, reverse primer: 5′- CAC TTC CAC GCC CAA GGC CCC-3′, product: 80 bp), and only samples positive for PBGD transcripts were used for EGFRvIII PCR. Conditions for the EGFRvIII PCR reaction and primers used therein were the same as described by Yoshimoto and colleagues (21).

Statistical analysis

The threshold for differentiating between low and high IHC scores was set at 7 or more for EGFR and EGFRvIII and at 4 or more for AREG, respectively. These optimal cutoff values for discrimination of patient subgroups with significantly different disease control rates (DCR) were determined by using the receiver operating characteristic curve analysis (see Supplementary Fig. S1), and the same cutoff values were subsequently applied to the analysis of PFS and OS. Comparison of PFS and OS between the patient subgroups with low or high expression score for EGFR, EGFRvIII, or AREG was done using the Kaplan–Meier analysis, and significance was determined using the log-rank test. The relation between EGFR, EGFRvIII, and AREG immunostaining score and survival was evaluated using the Cox regression model. The interference of EGFR, EGFRvIII, and AREG expression with the clinical parameters sex, age, tumor localization, initial tumor stage, smoking, and alcohol history as well as the DCR [partial response (PR) and stable disease (SD)] was assessed using logistic regression. Statistical analyses were carried out using the Statview software (version 5.0.1, SAS Institute Inc.). The level of significance was set at $P < 0.05$.

Results

Expression and subcellular localization of EGFR, EGFRvIII, and AREG

The analysis of expression and subcellular localization of EGFR, EGFRvIII, and AREG revealed positive tumor samples in 94% (EGFR), 80% (EGFRvIII), and 81% of cases (AREG). Although EGFR was predominantly localized at the cellular membrane, EGFRvIII and AREG were mainly detected in the cytoplasm. AREG expression could be found both in the cytoplasm and in the nucleus of tumor cells. High expression levels of EGFR, EGFRvIII (IHC score ≥7), and AREG (IHC score ≥4) were detected in 73%, 17%, and 45% of SCCHN cases, respectively. No significant associations of EGFR, EGFRvIII, and AREG immunostaining scores with sex, age, tumor localization, initial tumor stage, smoking, and alcohol history were observed. Representative tumor samples with low and high IHC scores for AREG (left) and EGFRvIII (right) are presented in Figure 1A.

To validate the results from the IHC analysis of EGFRvIII using the L8A4 antibody, we analyzed 4 EGFRvIII-positive

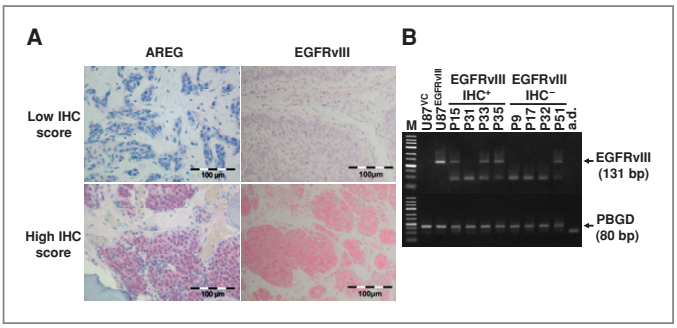

Figure 1. Detection of AREG and EGFRvIII in SCCHN. A, representative tumor samples with low and high IHC score for AREG (left) and EGFRvIII (right) are presented. B, EGFRvIII (top) or PBGD transcripts (bottom) were amplified by conventional RT-PCR. Products were separated by gel electrophoresis on a 3.5% agarose gel and detected by SybrGreen staining. From left to right: lane 1, 100-bp size marker; lane 2, the glioma cell line $U87^{VC}$; lane 3, $U87^{EGFRvIII}$; lanes 4–7, tumor samples positive for EGFRvIII in IHC; lanes 8–11, IHC-negative tumor samples; lane12, aqua destillata (a.d.).

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Table 1. Logistic regression analysis of molecular/clinical parameters and DCR after 3 months on cetuximab–docetaxel

Parameter	N	Univariate analysis DCR [%]	*P*	Multivariate analysis HR (95% CI)	*P*
EGFR			0.52		
Low IHC score	13	61			
High IHC score	20	50			
EGFRvIII			0.02		0.01
Low IHC score	37	65		1.0	
High IHC score	8	13		0.03 (0.002–0.44)	
AREG			0.09		0.12
Low IHC score	26	65		1.0	
High IHC score	20	40		0.3 (0.1–1.38)	
EGFR R521K			0.08		0.05
G genotype	22	68		1.0	
A genotype	24	42		0.19 (0.04–1.04)	
Skin toxicity			0.05		0.09
Any	33	64		1.0	
None	13	31		0.23 (0.05–1.26)	

samples with an IHC score of 6 or more, 4 negative FFPE samples with an IHC score of 0 and the U87 cell line transfected with a vector encoding for EGFRvIII ($U87^{EGFRvIII}$) or an empty vector ($U87^{VC}$) by reverse transcription PCR (RT-PCR). In 3 of 4 FFPE samples positive in the IHC analysis and $U87^{EGFRvIII}$ cells, a specific EGFRvIII PCR amplicon could be detected. Conversely, 3 of 4 EGFRvIII-negative FFPE samples and $U87^{VC}$ cells were negative in the EGFRvIII RT-PCR (Fig. 1B). Thus, the overall concordance between the 2 analyses was 80% which is equal to the value reported by Yoshimoto and colleagues (21).

EGFRvIII and AREG expression and outcome

Because EGFRvIII and AREG expression levels have been shown to negatively and positively interfere with the efficacy of cetuximab in SCCHN cell line models (13) and mCRC patient cohorts (14, 15), respectively, we first evaluated whether their expression would also correlate with the response to cetuximab–docetaxel in our study. Response data after 3 months were available for 46 of 47 patients. The overall response rate was 12% and the DCR (PR + SD) 54%. Although EGFR expression did not affect the DCR, there was a significant association of EGFRvIII expression and treatment efficacy: in the patient group with low EGFRvIII IHC score, DCR was 65%, whereas patients with high EGFRvIII IHC score had a DCR of 13% ($P = 0.02$; Table 1). Contrary to the mCRC model, where high AREG expression levels were associated with an improved response to cetuximab or cetuximab–irinotecan treatment, we observed a trend to reduced disease control in patients with high AREG IHC score (DCR 40%) as compared with patients with low AREG IHC score (DCR 65%, $P = 0.09$). As already reported recently (22), the A genotype of the EGFR single-nucleotide polymorphism R521K and the absence of any skin reaction in the early treatment phase were also by trend correlated with reduced response to cetuximab–docetaxel (Table 1). Therefore, these factors were also included in our multivariate model. Multivariate logistic regression analysis confirmed the independent significant association of EGFRvIII with response to treatment, whereas AREG expression levels, EGFR R521K, and skin toxicity remained only by trend associated with DCR (Table 1).

To further determine whether expression levels of EGFR, EGFRvIII, or AREG were significantly related to PFS and OS, patients were divided into low and high IHC score groups, again using the cutoffs of 7 or more for EGFR and EGFRvIII and 4 or more for AREG established for response to treatment. As depicted in Figure 2A, expression of EGFR had no impact on PFS and OS. In contrast, patients with tumors that showed high expression of EGFRvIII had significantly shorter PFS than patients with low expression (log-rank, $P = 0.0028$; HR: 3.3; mean PFS, 2.0 vs. 5.4 months; Fig. 2B), whereas OS was not significantly affected by EGFRvIII expression levels. Patients with tumors with high AREG expression had significantly shortened PFS (HR: 2.2, log-rank $P = .016$, mean PFS, 3.1 vs. 5.9 months) and OS (HR: 2.2, log-rank $P = 0.0016$, mean OS 5.5 *vs* 9.5 months) compared with patients with low AREG expression score (Fig. 3). Multivariate Cox models confirmed AREG, EGFRvIII, and skin toxicity as independent predictors for PFS (Table 2) but only AREG expression levels were identified as an independent predictor for OS (Table 3).

Discussion

We show that the majority of recurrent or initially metastatic SCCHN tumors express EGFR, EGFRvIII, and

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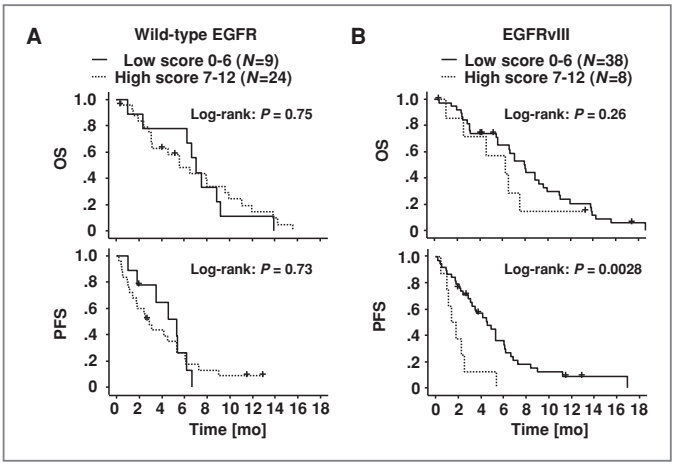

Figure 2. EGFRvIII but not wild-type EGFR expression levels interfere with PFS but not OS of SCCHN patients treated with cetuximab–docetaxel. Kaplan–Meier curves for OS (top) and PFS (bottom) of patients with low or high IHC score for wild-type EGFR (A) and EGFRvIII (B) are shown. *P* values for comparison of groups using the log-rank test are given.

AREG. The major fraction of AREG was detected in the cytoplasm and the nucleus of tumor cells. It has previously been shown that after ectodomain cleavage from its membrane-anchored precursor (23), soluble AREG binds to its receptor which results in the internalization of the receptor–ligand complex (24). Thus it seems likely that in our study mainly receptor-bound internalized AREG was detected. Comparable with the staining pattern of AREG, EGFRvIII was also mainly detected in the cytoplasm. This is also in line with previous results in glioblastoma where even under nonstimulated conditions, EGFRvIII was primarily found in the cytoplasm (25). In the latter study, constitutive tyrosine phosphorylation of EGFRvIII was identified as the molecular mechanism leading to the translocation of EGFRvIII from the membrane to the cytoplasm (25). These data, together with our results, are suggestive of the EGFR signaling pathway being constitutively activated in recurrent or initially metastatic SCCHN.

Our study showed for the first time that high AREG expression in SCCHN tumors is an independent prognosticator of poor outcome on cetuximab–docetaxel treatment. This is in clear contrast to the results from clinical studies of mCRC (14, 15) and NSCLC (26), in which the clinical activity of treatment with the EGFR targeting agents cetuximab, gefitinib, or erlotinib was positively associated with AREG expression. Technical differences in the AREG immunostaining methods as a possible reason for differences in the prognostic value of AREG expression levels are rather unlikely because the same antibody was used in the NSCLC (26) and our study. Furthermore, although AREG mRNA and not, as in our study, protein levels were determined in mCRC (14, 15), a good correlation between mRNA and protein expression levels, at least in NSCLC

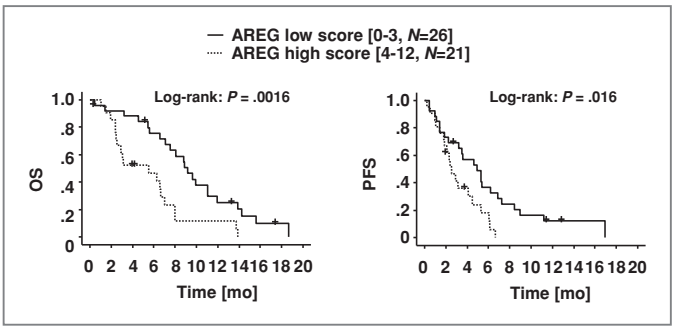

Figure 3. High AREG expression negatively influences OS and PFS of SCCHN patients treated with cetuximab–docetaxel. Kaplan–Meier curves for OS (left) and PFS (right) of patients with low and high IHC score for AREG are presented. *P* values for comparison of groups using the log-rank test are given.

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Table 2. Cox regression model for the interaction between molecular/clinical parameters and PFS

		Univariate analysis		Multivariate analysis	
Parameter	**N**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
EGFR			0.63		
Low IHC score	13	1.0			
High IHC score	20	1.2 (0.6–2.5)			
EGFRvIII			0.005		0.002
Low IHC score	37	1.0		1.0	
High IHC score	8	3.3 (1.4–7.5)		4.1 (1.7–10.0)	
AREG			0.019		0.015
Low IHC score	26	1.0		1.0	
High IHC score	20	2.2 (1.1–4.4)		2.5 (1.2–5.1)	
EGFR R521K			0.12		0.30
G genotype	22	1.0		1.0	
A genotype	24	1.6 (0.9–3.0)		1.4 (0.7–2.8)	
Skin toxicity			0.02		0.011
Any	33	1.0		1.0	
None	13	2.2 (1.1–4.5)		2.7 (1.2–5.6)	

and SCCHN cell line models, has been reported (17). Our results, therefore, suggest that differences in the biological function of AREG depending on the type of epithelial tissue from which the tumor originates might be responsible for the observed differences in the predictive value of AREG expression.

The assessment of the biological functions of AREG in animal models revealed moderate basal expression of AREG in normal gastrointestinal mucosa, which is upregulated after infection with *Helicobacter pylori* (27) or nematode parasites (28). During the transformation process, tumor cells derived from the intestinal mucosa (e.g., CRC) might have not only preserved the expression of AREG as tissue-protective factor but might even have upregulated its expression to acquire a survival advantage. Indeed, high AREG expression has been reported in CRC where it correlated with poor outcome (29). It is tempting to speculate that AREG-expressing tumor cells may become dependent on the constitutive activity of the EGFR signaling pathway. Such a mechanism, termed *pathway addiction*, has indeed been proposed as a possible explanation for the positive association of AREG expression with the efficacy of cetuximab treatment in mCRC (15). In line with the peculiar role of EGFR signaling in gastrointestinal mucosa,

Table 3. Cox regression model for the interaction between molecular/clinical parameters and OS

		Univariate analysis		Multivariate analysis	
Parameter	**N**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
EGFR			0.73		
Low IHC score	13	1.0			
High IHC score	20	1.1 (0.5–2.4)			
EGFRvIII			0.27		
Low IHC score	37	1.0			
High IHC score	8	1.6 (0.7–4.0)			
AREG			0.002		0.005
Low IHC score	26	1.0		1.0	
High IHC score	20	2.8 (1.5–5.5)		2.8 (1.4–5.7)	
EGFR R521K			0.10		0.79
G genotype	22	1.0		1.0	
A genotype	24	1.7 (0.9–3.2)		1.1 (.5–2.5)	
Skin toxicity			0.06		0.16
Any	33	1.0		1.0	
None	13	1.9 (0.9–3.8)		1.7 (.8–3.8)	

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there is also the clinical observation that cetuximab given concurrently with or after an aggressive induction chemotherapy such as docetaxel, cisplatin, 5-fluorouracil (TPF; ref. 30 and results from the EORTC24061, orally presented by Dr. Vermorken, Athens, November 2010) can result in severe and life-threatening gastrointestinal toxicities including mucositis, enteritis, and diarrhea. Such dose-limiting toxicities have never been reported for the mucosa in the head and neck region nor have we observed such effects on oral mucosa in our own ongoing clinical trials.

Such a difference in the biological role of EGFR signaling in different epithelial mucosa types, however, cannot explain the discrepant results on the role of AREG as a predictor of gefitinib or erlotinib treatment in NSCLC. High AREG protein expression was significantly associated with prolonged PFS and OS in a cohort of 73 NSCLC patients treated with gefitinib or erlotinib (26); however, increased AREG levels in plasma (31), serum (32), or tumor tissue (33) did not interfere with treatment efficacy or were associated with progressive disease on EGFR targeting in 3 further studies, although in all 4 study populations, the proportion of adenocarcinoma/squamous cell carcinoma was comparable. Certainly, additional studies are needed to understand the molecular basis for the different role of AREG for the prediction of treatment efficacy in CRC, NSCLC, and SCCHN.

In our study, the expression of wild-type EGFR did not interfere with the outcome, which is in line with results from studies combining cetuximab with platinum-based chemotherapy (9) or radiotherapy (7). However, we identified the expression of its deletion variant, EGFRvIII, as an independent prognostic factor of the DCR and PFS on cetuximab–docetaxel. Our data corroborate results from SCCHN cell line models in which ectopic expression of EGFRvIII not only increased tumor cell proliferation *in vitro* and tumor growth in a xenograft model but also rendered tumor cells more resistant to cisplatin and cetuximab treatment (13). Lower sensitivity of EGFRvIII-positive tumors to cisplatin treatment might also explain the higher frequency of EGFRvIII detection in our study compared with the study of Sok and colleagues (13), considering that only patients who relapsed after cisplatin-containing first-line treatment were eligible for our study.

Given that EGFRvIII also negatively interferes with radiosensitivity (34), all 3 major cornerstones of SCCHN treatment are potentially compromised by EGFRvIII expression, which makes the development of novel strategies for this unfavorable patient group an urgent clinical need. Because EGFRvIII is expressed exclusively by tumor cells, targeting of EGFRvIII may represent an attractive strategy to improve the clinical efficacy of chemoradiation as well as cetuximab-containing regimens without increasing the side effects of such multimodal treatment regimens. A specific antibody to EGFRvIII conjugated with cytotoxic compounds is currently being developed and has already proven efficacious in animal models of glioblastoma (35). Alternatively, SCCHN patients with EGFRvIII-positive tumors might benefit from next-generation tyrosine kinase inhibitors like HKI-272, which, when compared with gefitinib or erlotinib, showed a 100-fold higher potency in inhibiting the growth of EGFRvIII-transformed lung tumor cancer cells *in vitro* and *in vivo* (36).

In conclusion, high expression levels of both AREG and EGFRvIII were associated with reduced efficacy of cetuximab–docetaxel treatment in recurrent or initially metastatic SCCHN. Because of the small cohort size and the nonrandomized design of our clinical study, the predictive values of AREG and EGFRvIII remain undetermined. Prospective clinical evaluation of these biomarkers and their plasticity over time in larger patient cohorts will be necessary to establish whether or not expression of EGFRvIII and AREG can be used for tailoring treatment or for selecting patients for novel treatment strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Patienten abgeleitete Xenograftmodelle von Kopf-Hals Karzinomen

Klinghammer K, Raguse JD, Plath T, Albers AE, Joehrens K, Zakarneh A, et al. **A comprehensively characterized large panel of head and neck cancer patient-derived xenografts identifies the mTOR inhibitor everolimus as potential new treatment option**. Int J Cancer. 2015;136(12):2940-8

Die vorangegangenen Arbeiten an Formalin fixiertem Gewebe hatten die Analyse einzelner Marker erlaubt, jedoch war ein Vergleich zwischen behandelten und unbehandelten Tumorgewebe nicht möglich. Darüber hinaus waren RNA Expressionstudien nur von sehr wenigen Proben möglich, einerseits aufgrund der geringen Materialmenge und andererseits der zum Teil langjährigen Lagerung der Tumorblöckchen, die zu einer zunehmenden Degradierung der Nukleinsäuren führte. Um diese Limitationen langfristig zu überwinden, etablierten wir bis zur ersten Veröffentlichung Xenograftmodelle von 52 Patienten mit Kopf-Hals Tumoren. Patienten, bei denen im Rahmen der Diagnosestellung oder kurativ intendierten Operation die Entnahme von Tumorgewebe erfolgte, wurden in die Studie eingeschlossen. Binnen 24 Stunden erfolge die Transplantation von Tumorgewebe zusammen mit Wachstumsfaktoren und Matrigel subkutan in immundefiziente Nacktmäuse. Für knapp 50% der Tumore gelang die Etablierung im Sinne eines dauerhaften Tumorwachstums im Mausmodell. Innerhalb der ersten und zweiten Tumorpassage kam es im Zuge der Etablierung zum gelegentlichen Wachstumsstopp, so dass eine weitere Verwendung dieses Tumormodells nicht möglich war. Die dritte Passage wurde für Medikamententestungen verwendet. Evaluiert wurden die Tiere mit 5 FU, Platin, Cetuximab, Everolimus, Docetaxel und MTX behandelt. Der initiale Ansatz, ein Modell zur Patienten-individuellen Medikamententestung zu etablieren, gelang nicht, da nur für 50% der Patienten ein Modell etabliert werden konnte, die Tumore ihre Wachstumskinetik im Modell beibehalten und die Etablierung bis zur Medikamententestung durchschnittlich ein Jahr dauerte. Weiterhin sahen wir in der Auswertung der Überlebensdaten der Patienten einen signifikanten Zusammenhang von dem Versterben der Patienten an ihrem Tumor und erfolgreichen Anwachsen des Tumors im Modell, was die Tumorbiologie widerspiegelte. Daraus ließ sich schlußfolgern, dass aggressive Tumore im Tiermodell besser anwachsen, aber z.B. HPV positive Tumore, die eine sehr gute Prognose haben, nur sehr selten angewachsen sind. Bedeutsam waren umfangreiche molekulare Analysen der PDX

Tumore, in denen wir zeigen konnten, dass z.B. das Mutationsprofil im Tiermodell erhalten bleibt. Auch morphologisch waren PDX in der histopathologischen Begutachtung durch den Pathologen vom Primärtumor nicht zu unterscheiden. Nach Etablierung und umfangreicher Charakterisierung der Modelle erfolgten eine Reihe von Medikamentenstudien mit dem Ziel neue Medikamente für die Tumorentität der Kopf-Hals Karzinome zu evaluieren. Diese Untersuchungen wurden stets in molekular charakterisierten PDX-Modellen durchgeführt, um potentiell prädiktiver Biomarker zu identifizieren. In der ersten Arbeit konzentrierten wir uns auf die Inhibition von mTOR. Auch wenn die Ergebnisse für diese Substanzklasse der mTOR Inhibitoren nur moderat ausfielen, hatten wir eine Plattform geschaffen, die in Bezug auf die Kopf-Hals Karzinome bis heute die weltweit größte Sammlung von PDX darstellt und damit die Grundlage für weitere präklinische Studien ist.

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A comprehensively characterized large panel of head and neck cancer patient-derived xenografts identifies the mTOR inhibitor everolimus as potential new treatment option

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Patient-derived xenograft (PDX) models have shown to reflect original patient tumors better than any other preclinical model. We embarked in a study establishing a large panel of head and neck squamous cell carcinomas PDX for biomarker analysis and evaluation of established and novel compounds. Out of 115 transplanted specimens 52 models were established of which 29 were characterized for response to docetaxel, cetuximab, methotrexate, carboplatin, 5-fluorouracil and everolimus. Further, tumors were subjected to sequencing analysis and gene expression profiling of selected mTOR pathway members. Most frequent response was observed for docetaxel and cetuximab. Responses to carboplatin, 5-fluorouracil and methotrexate were moderate. Everolimus revealed activity in the majority of PDX. Mutational profiling and gene expression analysis did not reveal a predictive biomarker for everolimus even though by trend *RPS6KB1* mRNA expression was associated with response. In conclusion we demonstrate a comprehensively characterized panel of head and neck cancer PDX models, which represent a valuable and renewable tissue resource for evaluation of novel compounds and associated biomarkers.

Cancer Therapy

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer world wide.[1] Despite improved survival, especially through the introduction of targeted agents it remains a devastating disease. A potential patient stratification by means of predictive biomarkers has not been successfully established for clinical routine use.[2] The majority of anticancer drugs tested in early clinical trials failed to show a clinical benefit. Although preclinical drug evaluation in cell lines has been a useful tool for mechanistic exploration, those cell lines have repeatedly failed to predict clinical impact[3] and cell lines of HNSCC, especially HPV positive lines have proven difficult to establish. Patient-derived xenografts (PDX) have been recognized to better predict clinical outcome, since this preclinical model shares similar histology, comparable gene expression patterns over several passages and retain tumor heterogeneity as seen in the primary specimen.[4–6] We aimed to establish an extensive number of patient-derived xenograft models of HNSCC for translational research, preclinical drug screening and biomarker identification and validation. In a first step we characterized the established models for compounds used in standard of care (SoC) treatment to identify resistant tumors needing alternative treatment options and reanalyze response to SoC for predictive signatures in the available gene expression and mutational patterns. EGFR inhibition has become an important part of HNSCC treatment schedules, however still with limited success. Aim of our translational studies was the search for a rational alternative targeted treatment. Everolimus is one of two mTORC1 inhibitors, which have been successfully introduced into clinical routine for the treatment of renal cell carcinoma, breast cancer and neuroendocrine tumors. However, not all patients experience a benefit from mTOR inhibition and biomarker identification for patient selection has been defined as a crucial issue.[7] We aimed to evaluate the established PDX models for treatment response to everolimus and correlate our findings with tumor biology. By this way

Key words: xenograft, head and neck cancer, PDX, everolimus, PI3K, patient avatar, mTor, HNSCC
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What's new?
Preclinical drug evaluation in head and neck squamous cell carcinoma (HNSCC) is challenged by the inability of established cell lines to predict clinical impact. It may be possible to overcome that problem with patient-derived xenografts (PDX), which more closely reflect tumor characteristics. Here, a large collection of PDXs were established for HNSCC and tested for therapeutic response. The mTOR inhibitor everolimus was found to be active in a majority of the models. Biomarkers capable of predicting tumor response to everolimus were not identified, though increased expression of *RPS6KB1*, a member of the mTOR pathway, was common among responders.

we intended to develop a well-founded hypothesis for clinical application of this compound.

Material and Methods

Establishment of patient-derived xenografts

Patients with head and neck tumors planned for surgical treatment were approached for sample donation. Patients included in the study stated written informed consent and the study was approved by the local Institutional Review Board of Charité University Medicine, Germany (EA4/019/12). Tumor samples, which were not needed for pathological review were used for xenotransplantation. Tumor pieces of 3–4 mm were placed in RPMI media and transferred at room temperature to the animal facility. Transplantation was done on NOD.Cg-Prkdcscid *Il2rgtm1Wjl/SzJ (NSG)* mice subcutaneously within 24 hr after tumor surgery, since these mice have been advocated for the highest engraftment rate compared with other strains.[8] Additional tissue samples were immediately snap-frozen and stored at −80°C for genomic and protein analyses. All animal experiments were done in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals and of the German Animal Protection Law and approved by the local responsible authorities.[9] Samples were anonymized and given an internal number. In case of transplantation of primary tumor and metastasis from the same patient, this was indicated by A and B, respectively.

Engrafted tumors at a size of about 1cm^3 were surgically excised and smaller fragments retransplanted to naïve NMRI nu/nu mice for further passage. Next to economical consideration this strain was chosen since engrafted tumors will continue growth on less immunocompromised mouse strains and to ensure comparability of results since maximum tolerated doses was previously assessed in nude mice within our group. Within passage 1 to 3 numerous samples were conserved in DMSO for further experiments. Tumors were passaged not more than six times.

Chemosensitivity testing

Response to compounds used in clinical routine was evaluated in early passages after confirmation of histological tumor identity. For determination of chemotherapeutic response fragments of similar size were transplanted subcutaneously to a large cohort of mice. At palpable tumor size (50–100 mm^3), mice were randomized to a treatment or control group consisting of six animals each. Doses and schedules were chosen according to previous experience in animal experiments and represent the maximum tolerated or efficient doses. Applied schedules are shown in Table 1. The injection volume was 0.2 ml/20 g body weight. Treatment was continued over a period of 3 weeks unless tumor size exceeded 2 cm^3 or animals showed loss of 10% body weight. No group lost more than one animal due to toxicities during the treatment. At the end of the treatment period animals were sacrificed and tumor samples were stored in liquid nitrogen immediately.

Tumor evaluation

Animals were observed twice daily for health condition. Twice weekly, animals were evaluated for tumor size and body weight. Tumor measurement was done two-dimensional with a sliding caliper. Individual tumor volumes (V) were calculated by the formula: $V = ([\text{width}]^2 \times \text{length})/2$. Mean tumor volumes of treated in relation to mean tumor volume of control animals (T/C) were used for the sensitivity evaluation of each treatment modality after 3 weeks of treatment.

H&E staining of primary tumor and xenografts

For confirmation of tumor histology tumor tissue was embedded in Tissue-tek and 5 μm cryo sections were prepared. Samples were stained according to a standard protocol for hematoxilin eosin to ensure xenograft comparability to the original specimen. Cases with changed histological pattern were sent for pathological review and CD 20 staining was performed in order to exclude the outgrowth of lymphoproliferative disorders.

Determination of HPV status

P16 staining as surrogate marker for HPV infection was used for screening at the pathology department of Charité University Hospital on patient tumor material. To confirm and asses stable expression of HPV DNA in tumor xenografts PCR analysis for E6 and E7 was performed in p16 positive cases. Analysis was restricted to HPV-16 since this type comprises about 90% of HPV associated tumors in the oropharynx.[10] Primers and probes used were adapted from Zhao *et al.*[11] Total genomic DNA was isolated from tumor samples using DNeasy blood and tissue kit from Qiagen according to the

manufacturer's instructions. Quality and quantity assessment was accomplished using Nanodrop Spectrophotometer 1000. All samples were run in duplicate.

RNA preparation and quantitative PCR

RNA isolation was done using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Quality and quantity assessment was accomplished using Nanodrop Spectrophotometer 1000 (PeqLab). RNA was reverse transcribed using SuperScript III Reverse Transcription Kit (Invitrogen). Human gene primer/probe pairs for *MTOR* (Gene ID 2475, HS00234508), *RPS6KB1* (Gene ID 6198 HS00177357), *Akt1* (Gene ID 207, HS00178289), *FKBP1B* (Gene ID2281, HS00997682) *TSC1* (Gene ID 7248, HS1060648) and *GAPDH* (Gene ID 2597 HS99999905) and TaqMan Fast Master Mix obtained from Applied Biosystems were used according to the manufacturer's instructions and amplifications were carried out on the Applied Biosystems 7500 Real-time PCR cycler. GAPDH was used as housekeeping gen.

All samples we run in duplicate. Results are displayed as delta CT values as relative quantification.

Table 1. Compounds, dosage, application schedule and route of application of evaluated substances in patient derived xenografts

Compound	Dose	Schedule	Application route
Docetaxel	12.5 mg kg^{-1}	Once weekly x3	iv
Carboplatin	75 mg kg^{-1}	Once weekly x3	ip
Cetuximab	50 mg kg^{-1}	Once weekly x3	iv
5-fluorouracil	100 mg kg^{-1}	Once weekly x3	ip
Methotrexate	10 mg kg^{-1}	q3d	ip
Everolimus	4 mg kg^{-1}	d1–5 x3 weeks	po

Abbreviations: iv: intravenous, ip: intraperitoneal, po: per os, q3d: every 3rd day.

DNA Sequencing

Mutational analysis of primary tumor samples and selected corresponding xenografts was accomplished on Illuminas TruSeq Amplicon—Cancer Panel. With this panel 48 genes are targeted with 212 amplicons in a multiplexed reaction.

Table 2. Clinical characteristics of patients whose tumors led to successful establishment of patient derived xenografts

Tumor ID	TNM	UICC stage	Grading	Age	Site of tumor origin	Gender	Primary/recurrent
9619	T2N0M0	II	NA	NA	Oropharynx	Female	Recurrent
9876	T3N2cM0	IVA	G3	62	Hypopharynx	Male	Recurrent
9897	T2N2bM0	IVA	G3	58	Hypopharynx	Male	Recurrent
10110	T2N2cM0	IVA	G2	69	Tongue	Male	Primary
10114	T3N0M0	III	G3	52	Floor of mouth	Male	Primary
10159	T1N0M0	I	G2	57	Floor of mouth	Male	Primary
10309	T4N2cM0	IVA	G3	55	Oropharynx	Male	Primary
10321	T2N0M0	II	G2	65	Tongue	Male	Primary
10379	T3N2bM0	IVA	G2	39	Soft palate	Male	Primary
10511	T2N0M0	II	G2	54	Oropharynx	Male	Primary
10621	T2N2bM0	IVA	G3	61	Oropharynx	Male	Primary
10632	T2N1M0	III	G3	60	Tongue	Male	Primary
10847	T2N1M0	III	G2	71	Soft palate	Female	Recurrent
10913	T4N2bM0	IVA	G2	50	Floor of mouth	Male	Primary
10924	T3N2cM0	IVA	G2	65	Hypopharynx	Male	Primary
10927	T2N2bM0	IVA	G2	67	Oropharynx	Male	Primary
10960	T2N0M0	II	G2	63	Tongue	Male	Primary
10980	T4bN2bM0	IVB	G2	59	Soft palate	Female	Primary
11097	T4aN2bM0	IVA	G2	75	Floor of mouth	Female	Primary
11142	T2N2cM0	IVA	G3	46	Floor of mouth	Male	Primary
11143	T2N2bM0	IVA	NA	82	Oropharynx	Male	Primary
11218	T4N0M0	IVA	G2	68	Soft palate	Female	Primary
11269	T4aN2cM0	IVA	G2	71	Floor of mouth	Male	Primary
11437	T4bN2cM0	IVB	G2	56	Floor of mouth	Male	Primary
11452	T2N0M0	II	G2	75	Floor of mouth	Male	Primary
11482	T2N2bM0	IVA	G2	61	Floor of mouth	Male	Primary

All necessary reagents were purchased from Illumina. Total genomic DNA was isolated from tumor samples using DNeasy blood and tissue kit from Qiagen according to the manufacturer's instructions. About 250 ng of high quality genomic DNA (A260/280 1.8-2) were used for hybridization to a custom pool of oligos on a hybridization plate. Unbound oligos were removed using a filter capable of size selection by repeated washing followed by extension-ligation of bound oligos, which resulted in the formation of products containing the targeted regions of interest. The products were amplified using primers that add index sequences for sample mulitplexing as well as common adapters required for cluster generation. This was followed by library normalization. For cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer and heat denatured prior to MiSeq sequencing. MiSeq sequencing was carried out on Illumina MiSeq. Illumina Variant Studio 2.1 was used for sample analysis. For correlation analysis, known SNPs were excluded and only somatic mutations, which occurred with an allelic frequency >5% were considered. We have to acknowledge employing chip technology in sequencing analysis bears the risk of missing genetic variants that might occur to a minor extend in other regions of the genome as covered by the amplicons prespecified.

Primary tumors and thereof derived xenografts were evaluated in nine matched samples. For the all others, tumor DNA was isolated from the tumors of the control group animals in the chemosensitivity evaluation.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5. Response evaluation after 3 weeks of treatment using *T*/*C* values was done by two way ANOVA testing. A p value of <0.05 was considered as statistical significant. Correlation analysis was performed as Spearman rank-order correlation with a two tailed p value.

Results

In total 115 tumor samples from 89 patients with primarily diagnosed or recurrent head and neck squamous cell carcinomas were transplanted to immunodeficient mice. About 52 (45%) led to stable growth with confirmed histological appearance, whereas 63 (55%) did not grow after transplantation or resulted in outgrowth of CD20 positive lymphoproliferative disease ($n = 10$) not resembling the primary tumor. Median time to first passage was 69 days after tumor inoculation, ranging from 30 to 222 days.

From all cases, 14 tumors were classified as HPV positive by strong p16 expression in the majority of cancer cells. However, we successfully established only two PDX models (14% engraftment rate) from these HPV positive tumors. Interestingly we observed a high rate of lymphoproliferative disease in six HPV positive tumors.

PCR for viral genome of HPV type 16 showed stable expression of E6 and E7 over several xenograft generations for the two stable growing models. Twenty-nine models of HNSCC PDX were used for preclinical drug sensitivity screening after reaching 3rd passage. Patient characteristics of established PDX are summarized in Table 2. The number of models represents a clinical phase II setting considering a probability of response of 0.2 according to Simon *et al.*[12] and thereby provide representative data about single agent activity. For various tumors tissue from corresponding local lymph node metastases were transplanted. To date this resulted in three models for which we successfully established paired PDX of primary tumor as well as metastatic disease.

Validation studies

Several analyses were performed to verify, that the tumor growing in the PDX resembled the tumor characteristics found in the corresponding patient. Hematoxillin & Eosin (H&E) staining revealed high similarity of PDX and primary patient tumor, however, we observed 10 cases of changed histological pattern from squamous cell carcinoma to lymphoblastic disease in the entire cohorte. Those cases were confirmed by human specific CD20 staining as lymphoblastic cells and excluded from further analysis.

We performed NGS using the Illumina Cancer panel to identify mutational spectrum of the new models. By sequencing both, the patient tumors and the engrafted PDX we were able to follow up on how mutational patterns are consistent over several passages in patient-derived xenografts models of head and neck cancer. Figure 1 shows mutations in the majority of samples.

Mutational analysis of our cohort on the Illumina Cancer Panel revealed a mutational pattern comparable to the recently described panel of Stransky *et al.* and the large cohort evaluated within the TCGA.[13,14] We detected TP53 mutation in 20 of the 29 (69%) models and PIK3CA mutation in seven models (24%) of the cohort. Other mutations occurred with low frequency. The majority of TP53 mutations were classified as deleterious, according to the functional evaluation by Kato *et al.*,[15] which is shown in the Supporting Information.

Chemosensitivity studies

Response to treatment was very heterogenous. *T*/*C* value below 50% and significant growth inhibition to control tumors were considered as responder. Overall best response rate was observed for treatment with docetaxel with 26 of 29 (89%) responders (mean *T*/*C* value of 23) and by cetuximab with 23/29 (79%) responders (mean *T*/*C* value of 32). Even though the dosage of classical chemotherapies such as 5-fluorouracil, carboplatin and methotrexate was according to maximum tolerated dose, in general this did not result in significant growth inhibition when given as single agent as in our approach. Responders for 5-FU were 14/29 (48%), for carboplatin 13/29 (44%) and methotrexate 6/29 (20%) with mean *T*/*C* values of 59, 62 and 82 respectively. Representative study results are shown in Figure 2.

Additionally to standard of care compounds we evaluated the mTOR inhibitor everolimus. Response rate was 20/29

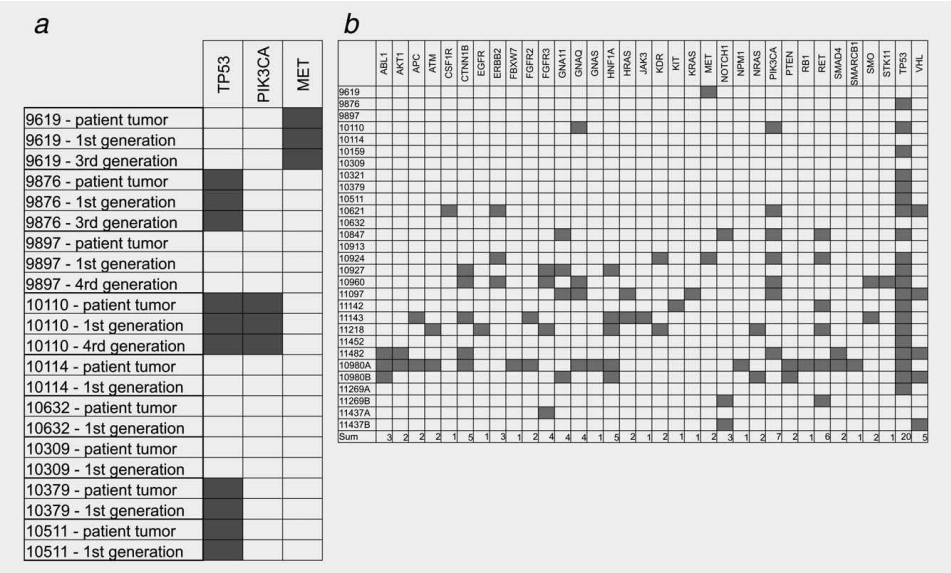


Figure 1. (*a*) showing mutational profile consistency over multiple generations for nine different patient tumors and thereof derived xenograft models. Model 10309 without any detectable mutation was identified as HPV positive. (*b*) showing the mutational profile of the entire characterized PDX panel as detected on Illumina Cancer Panel.

(68%) with a mean *T*/*C* value of 50. *T*/*C* values are shown in Figure 3.

Functional mTOR pathway analysis and correlation of response to mutational patterns

To correlate basal expression of the key members of the mTOR pathway, we analyzed quantitatively the gene expression with RT PCR. Figure 4 shows the delta CT Expression values of Akt1, mTOR, and RPS6KB1. (TSC1 and FKBP1B expression levels can be found in the Supporting Information). RPS6KB1 gene expression showed a trend to positive correlation with treatment response (T/C) values ($p = 0.0784$). Expression level of mTOR was significantly associated to expression of AKT1 ($p = 0.003$), TSC1 ($p = 0.0012$) and RPS6KB1 ($p = 0.0064$) but not to FKPB1B ($p = 0.7958$).

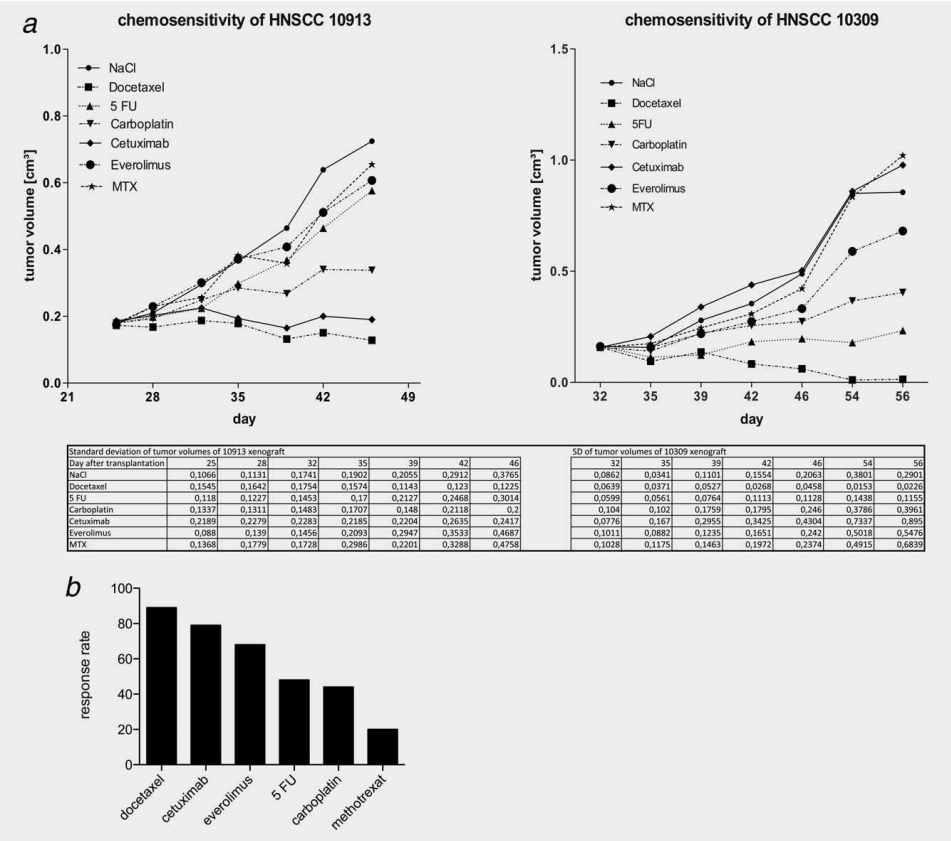
We observed the highest expression of mTOR pathway genes within the models 10110, 10980B and 11097. However, high gene expression levels of mTOR pathway members did not clearly translate into a better response to everolimus compared to models with low expression of RPS6KB1, Akt1 and mTOR such as 11142, 11482 and 11437A.

The detection of TP53 mutation did not influence treatment response to everolimus, independent whether mutations were classified as deleterious or tolerable. PIK3CA mutation has been reported to occur in up to 20% of head and neck carcinomas and activating mutations have been associated to increased pathway signaling, tumor formation and sensitivity toward PI3KCA inhibitors.[16,17] However, there was no statistical significant correlation in gene expression within the evaluated mTOR pathway and the occurrence of PI3KCA mutation as well as response to Everolimus in our models.

Discussion

Within 2 years we successfully established 52 patient-derived xenografts of head and neck squamous cell carcinoma, which to our knowledge represents the largest collection of this tumor entity. Employing PDX for biomarker studies and evaluation of new treatment modalities has been advocated as a superior preclinical model in comparison to cell lines because those models reflect the original patient tumor closer than any other preclinical model.[18–20] Furthermore, the collection of different patient tumors on xenografts reflects the diversity of HNSCC.

As others before, we were able to show that histological patterns are resembled in the xenograft tumor.[17,21,22] Furthermore we were able to show that mutational patterns are conserved over several passages within our validation studies. Even though a growing body of evidence shows similarity between original patient tumor and thereof derived xenograft tumors thorough validation for each patient-derived xenograft remains an essential issue, since patient tumor



Standard deviation of tumor volumes of 10913 xenograft							
Day after transplantation	25	28	32	35	39	42	46
NaCl	0,1066	0,1131	0,1741	0,1902	0,2055	0,2912	0,3765
Docetaxel	0,1545	0,1642	0,1754	0,1574	0,1143	0,123	0,1225
5 FU	0,118	0,1227	0,1453	0,17	0,2127	0,2468	0,3014
Carboplatin	0,1337	0,1311	0,1483	0,1707	0,148	0,2118	0,2
Cetuximab	0,2189	0,2279	0,2283	0,2185	0,2204	0,2635	0,2417
Everolimus	0,088	0,139	0,1456	0,2093	0,2947	0,3533	0,4687
MTX	0,1368	0,1779	0,1728	0,2986	0,2201	0,3288	0,4758

SD of tumor volumes of 10309 xenograft							
32	35	39	42	46	54	56	
0,0862	0,0341	0,1101	0,1554	0,2063	0,3801	0,2901	
0,0639	0,0371	0,0527	0,0268	0,0458	0,0153	0,0226	
0,0599	0,0561	0,0764	0,1113	0,1128	0,1438	0,1155	
0,104	0,102	0,1759	0,1795	0,246	0,3786	0,3961	
0,0776	0,167	0,2955	0,3425	0,4304	0,7337	0,895	
0,1011	0,0882	0,1235	0,1651	0,242	0,5018	0,5476	
0,1028	0,1175	0,1463	0,1972	0,2374	0,4915	0,6839	

Figure 2. (*a*) representative growth curves of two head and neck cancer patient-derived xenograft tumors. Treatment duration lasted for 3 weeks. One treatment group consisted of six animals. The right graph shows a HPV positive model. Underneath standard deviation of tumor volumes are given at time points of measurement. (*b*) response rate in percent for the evaluated compounds of 29 xenografts.

fragments which always include lymphocytes of the donor patient transplanted to NSG mice may result in outgrowth of transformed B-cells to form a type of lymphoproliferative disease. Within our cohort of 115 transplanted tumor samples we observed lymphoproliferative disease in 10% of transplanted samples of squamous cell carcinomas. Mouse lymphoma was ruled out by usage of human specific CD20 antibody. This phenomenon is in accordance to a report by Chen *et al.* who observed lymphoproliferative disease in 11 of 21 transplanted samples of hepatocellular carcinoma.[23] Before the introduction of NSG mice the phenomenon of evolution of lymphoproliferative disorder has not been an issue.[24] However employing NSG mice has led to superior engraftment rates and therefore remains a valuable platform with the need of thorough validation.[8]

Our collection of PDX lacks a relevant number of HPV positive tumors although a significant number of HPV positive tumors were initially transplanted. Even though it has been reported that HPV positive tumors show similar engraftment rates to HPV negative tumors, we were not able to reproduce this observation.[21] Because HPV associated tumors occur most often in tonsilar squamous cell epithelium, a per se lymphocyte rich tissue, xenotransplants from those tumors frequently gave rise to lymphoproliferative disease. Another reason for the low number of HPV positive models may arise from the study population, which were mainly elderly smokers with tumors in the oral cavity. However, for the p16 positive tumors it remains an unsolved issue why engraftment is low and it might be a similar problem as seen in the attempts of establishing HPV positive cell lines.

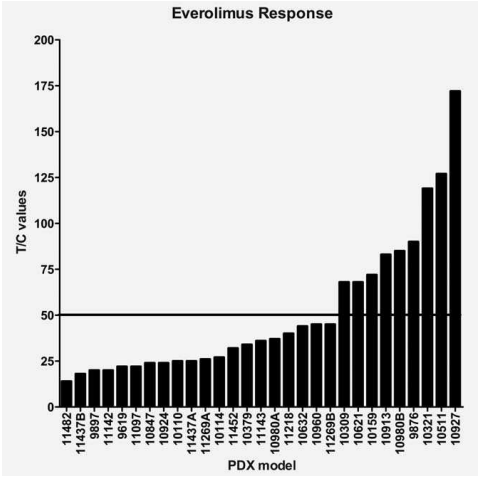


Figure 3. Everolimus response of individual patient-derived xenograft tumors expressed in *T/C* values. The line at *T/C* 50 marks the cut off for treatment responders and non responder. *T/C* values below 50 were considered as responders. Models indicated with A represent primary tumors and B the established model of the corresponding loco regional lymphatic metastasis.

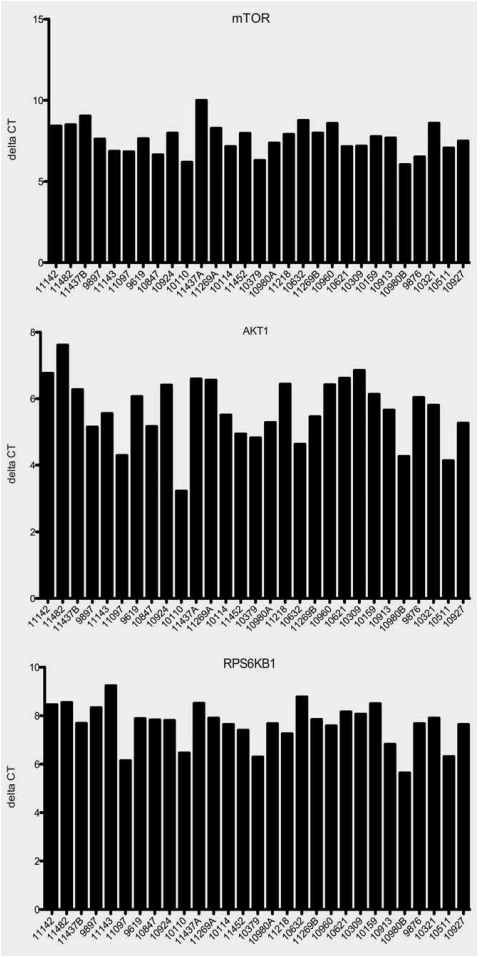


Figure 4. Delta CT values for mTOR pathway members RPS6KB1, AKT1 and mTOR sorted to Everolimus response with best responders on the left to resistant tumors on the right.

The two models, which we successfully established were positive for HPV16 and PDX tumors retained the HPV genome over several passages.

In our drug screening studies we evaluated the impact of traditionally used chemotherapeutic agents in head and neck cancer as a basis for further correlations, biomarker studies and definition of potential therapeutic partners. Highest response rate was observed for docetaxel (89%) and cetuximab (79%) and moderate activity for 5 FU (48%), methotrexate (20%) and carboplatin (44%). Prolonged treatment periods and combination treatments were beyond the scope of this study. The majority of the study population consisted of previously untreated patients, which might explain the high response rates of docetaxel and cetuximab. Appropriate *T/C* value cut off points for a clinical meaningful response is a controversial issue. Voskoglou–Nomikos proposed if more than one third of the evaluated animals show a meaningful response we might expect some activity in a phase II trial.[25] We choose to set the cut off point at *T/C* 50%. Johnson *et al.* evaluated different *T/C* values and found no differences by setting the response rate cut off point below 10% in contrast to below 40.[26]

As novel treatment option and to study possible predictive biomarkers we evaluated everolimus, a compound targeting the mTOR pathway. Dysregulation of the PI3K/Akt/mTOR pathway is a common event in the pathogenesis of head and neck cancer but mTOR inhibitors have not been introduced into clinical routine use in this tumor entity.[17,27] Preclinical evaluation of mTOR inhibitors in cell lines of head and neck cancer showed antiproliferative effects and induction of apoptosis.[28] Those results led to the CAPRA trial, a phase I/II trial exploring everolimus in combination with carboplatin and paclitaxel as induction regimen.[29] Temsirolimus, another mTOR inhibitor was evaluated in a phase II study design in recurrent/metastatic head and neck cancer, which showed moderate activity after failure of standard of care treatment.[30] In our experiments everolimus showed a significant growth inhibition in 20 of 29 (68%) head and neck cancer patient-

derived xenografts when given as single agent, which creates further evidence of activity of this compound in head and neck cancer. By extensive molecular profiling we aimed at identifying predictive markers for mTOR inhibition. Preclinical evaluation of activation of the PIK3/Akt/mTOR axis has been associated with response to inhibitors targeting this axis.[31] In our studies mRNA expression analysis of pathway members such as Akt1, mTOR, RPS6KB1, FKBP1B and TSC1 did not reveal a significant association between gene expression level and response to everolimus even though we observed a trend for higher RPS6KB1 expression ($p = 0.07$) within responders. Further analysis concentrated on evaluating mutational status focusing on PI3KCA, which has been reported to be associated with treatment response.[31] Within our head and neck PDX panel we observed 7 (24%) models with PI3KCA mutations, which resembles the frequency of PI3KCA mutation reported by TCGA in head and neck cancer.[14] It has been well established that PI3K mutation may lead to tumor formation and serves as predictive marker for novel inhibitors of PI3K.[17,32] We therefore explored whether mutational status was associated to everolimus response. According to Polivka *et al.* the most frequent mutation within PI3KCA is E545K,[32] which we found in three of our models (10621, 11097 and 11482). Another mutational hot spot (H1047R) is located in the kinase domain of the p110 alpha subunit, which we detected in tumor model 10110. Other less frequent mutations were detected in the model 10960 (E542K), 10924 (E542Q) and 10847(G1049R). All but one model (10621) harbouring mutations within the PI3K gene showed a significant growth inhibition (6/7,85%), when treated with everolimus but other models with PI3KCA wild-type (14/22, 63%) responded in a similar way. We therefore conclude that PI3KCA mutational status alone may not serve as a predictive marker for the stratification of patients to treatment with mTOR inhibitors. Additional biomarkers (*e.g.*, phosphoprotein assays for proteins of the pathway) or more complex combinations of biomarkers should be evaluated for their predictive power. It will be an interesting question, whether novel compounds targeting mTOR and PI3KCA together or PI3KCA alone act in dependency of the occurrence of this mutation in a heterogeneous tumors as seen in our models and in clinical routine. In conclusion, we observed a response in the majority of our PDX for the treatment with everolimus, which justifies further preclinical and clinical evaluation of this compound, and defining a predictive biomarker or a more complex biomarker pattern for patient selection remains an important goal for translational studies. Further studies using our newly established xenograft series will also concentrate on defining the best chemotherapeutic partner of everolimus and evaluation of novel targeted compounds in head and neck cancer.

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Der Stellenwert einer Phosphoarray Plattform zur Behandlungsstratifizierung

Klinghammer K, Keller J, George J, Hoffmann J, Chan EL, Hayman MJ. **A phosphoarray platform is capable of personalizing kinase inhibitor therapy in head and neck cancers**. Int J Cancer. 2018;142(1):156-64

Die Identifikation eines prädiktiven Biomarkers für den Einsatz von Cetuximab, die eine Patientenselektion erlauben würde, war zentrale Fragestellung der weiteren Arbeiten. Die etablierte PDX Plattform wurde verwendet, um das Ansprechen auf Cetuximab im Tumormodell zu ermitteln und diese Tumore im Anschluß zu charakterisieren. Mit Cetuximab behandelte und unbehandelte Tumore wurden nach der Behandlung für weitere Analysen bei -80°C archiviert. Bereits während der Gewinnung des Tumormaterial von den Tieren wurde stets auf eine rasche Prozessierung geachtet, um eine Degradierung von Signalproteinen gering zu halten. In Proteinlysaten wurde mittels eines vergleichsweise einfachen Testverfahrens die Phosphorylierung von 49 Rezeptortyrosinkinase evaluiert. In 35 von 39 untersuchten Tumoren zeigte sich eine Aktivierung des EGFR durch Nachweis einer Phosphorylierung. Fehlende Phosphorylierung war mit einer Resistenz gegenüber Cetuximab assoziiert. Weiterhin war eine Phosphorylierung von Her-2 mit einem schlechteren Therapieansprechen auf die alleinige EGFR Blockade assoziiert.

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A phosphoarray platform is capable of personalizing kinase inhibitor therapy in head and neck cancers

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Tyrosine kinase inhibitors are effective treatments for cancers. Knowing the specific kinase mutants that drive the underlying cancers predict therapeutic response to these inhibitors. Thus, the current protocol for personalized cancer therapy involves genotyping tumors in search of various driver mutations and subsequently individualizing the tyrosine kinase inhibitor to the patients whose tumors express the corresponding driver mutant. While this approach works when known driver mutations are found, its limitation is the dependence on driver mutations as predictors for response. To complement the genotype approach, we hypothesize that a phosphoarray platform is equally capable of personalizing kinase inhibitor therapy. We selected head and neck squamous cell carcinoma as the cancer model to test our hypothesis. Using the receptor tyrosine kinase phosphoarray, we identified the phosphorylation profiles of 49 different tyrosine kinase receptors in five different head and neck cancer cell lines. Based on these results, we tested the cell line response to the corresponding kinase inhibitor therapy. We found that this phosphoarray accurately informed the kinase inhibitor response profile of the cell lines. Next, we determined the phosphorylation profiles of 39 head and neck cancer patient derived xenografts. We found that absent phosphorylated EGFR signal predicted primary resistance to cetuximab treatment in the xenografts without phosphorylated ErbB2. Meanwhile, absent ErbB2 signaling in the xenografts with phosphorylated EGFR is associated with a higher likelihood of response to cetuximab. In summary, the phosphoarray technology has the potential to become a new diagnostic platform for personalized cancer therapy.

Imatinib is the first tyrosine kinase inhibitor (TKI) that directly targeted an oncogenic driver mutant. This drug showed unprecedented success in the treatment of chronic myeloegenous leukemia.[1] Since then, many kinase inhibitors targeting different oncogenic kinases were developed. A few of these drugs showed equally impressive efficacy, for instance, crizotinib for the non small cell lung cancers (NSCLC) that harbored the EML4-ALK translocation,[2] vemurafenib for the BRAF V600E mutated melanoma,[3] erlotinib for the NSCLC that harbored activating EGFR kinase mutations[4] or vandetanib for the hereditary medullary thyroid cancer with underlying RET mutation.[5] Like imatinib, the common theme around these success stories is that the TKIs specifically targeted the oncogenic mutants that drive the underlying cancers. Thus, recent effort has been focused on profiling the genetic landscape of tumors to identify

Key words: phosphoarray, head and neck squamous cell carcinoma, kinase inhibitors, personalized medicine, cetuximab response

Abbreviations: CR: complete response; DMSO: dimethyl sulfoxide; EGFR: epidermal growth factor receptor; ErbB2/HER2: avian erythroblastosis oncogene B/human epidermal growth factor receptor 2; HNSCC: head and neck squamous cell carcinoma; IGFR: insulin growth factor receptor; MET: hepatocyte growth factor receptor; NGS: next generation sequencing; NSCLC: non small cell lung cancers; p: phosphorylated; PD: progressive disease; PDGFR: platelet derived growth factor receptor; PDX: patient derived xenograft; PR: partial response; SD: stable disease; RTK: receptor tyrosine kinase; TKI: tyrosine kinase inhibitor

Additional Supporting Information may be found in the online version of this article.

K.K. and J.K. contributed to the work equally.

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What's new?
Advancing precision medicine has become a priority in many countries. The current protocol for personalized cancer therapy involves genotyping tumors in search of various driver mutations and selecting individual tyrosine kinase inhibitors accordingly. But although promising, the genotype approach has several limitations. Here, the authors show for the first time that a phosphoarray platform examining the activity of multiple kinases simultaneously is also capable of individualizing kinase inhibitor therapy in head and neck squamous cell carcinoma. The results provide the proof of concept that the phosphoarray technology can be further developed into a new diagnostic platform for personalized cancer therapy.

potential druggable targets, thereby increasing the efficacy of kinase inhibitor therapies.

With the rapid advance in sequencing technologies, high throughput screening of mutation drivers by next generation sequencing (NGS) is now a commercially available service for personalized cancer therapy. There are many anecdotal cases that utilized the NGS platform to identify driver mutations in cancer patients for novel TKI therapy.[6–10] In some cases, the diagnostic was successful in personalizing the right TKIs for the right patients. For instance, when a 41 year old woman with refractory, progressive sarcoma ran out of therapeutic options, NGS identified a novel TRK receptor fusion product, LMNA-NTRK1, in her original tumor. She was subsequently enrolled in a phase I trial of a new pan-TRK inhibitor, LOXO-101. After five cycles of LOXO-101, there was complete resolution of her metastatic diseases.[11] Similarly, after MET exon 14 mutations were identified in 0.6% of lung adenocarcinoma by NGS, three patients with tumors harboring these MET mutants were treated with MET directed therapies *via* clinical trials. All three demonstrated partial responses.[12]

Despite these success stories, the NGS platform has limitations as a personalized diagnostic. First, it might reveal many passenger mutations that are not drivers of the tumor. Second, bearing driver mutants does not necessarily translate into response to the corresponding TKIs. For instance, vemurafenib did not produce a dramatic response in the treatment of BRAF V600E mutated colorectal cancer.[13] Third, low mutation rates in some cancers like pediatric tumors[14] might limit the usefulness of NGS as a personalized diagnostic. Fourth, there might not be mutation drivers of a known target in the tumor. For example, EGFR is a known target for head and neck squamous cell carcinomas (HNSCC), but HNSCC rarely carried activating EGFR kinase mutations.[15,16] Finally, there might be other mechanisms of altering oncoprotein function/activity that NGS diagnostic is not able to identify. Such mechanisms might include overexpression, impaired degradation, defective negative feedback loop or constitutive activation. To improve the genotype approach, we hypothesized that a diagnostic that examine the activity of multiple kinases simultaneously might complement the NGS platform for better selection of the right patient for the right TKI.

Phosphoarray is a high throughput screening tool that examines the activities of multiple kinases simultaneously. One commonly used array is called the human phospho-receptor tyrosine kinase (RTK) array. Capture and control antibodies were spotted in duplicate on nitrocellulose membranes. When cell/tumor lysates were incubated with this array, both phosphorylated and unphosphorylated RTKs would bind. The active receptors would then be detected by chemiluminescence using a horseradish peroxidase conjugated anti-phosphotyrosine antibody. The phospho-RTK array can examine the phosphorylation status of 49 RTKs in the lysates simultaneously. While this array has been used in the laboratory setting to identify molecular pathway changes,[17–21] it has not been tested as a diagnostic to predict tumor response to TKI therapy. However, when it was used retrospectively to identify pathway changes in primary tumors, the array results seem to correlate with patient's response to the TKI, sunitinib.[22,23] In the four refractory thymic carcinoma patients who demonstrated response to sunitinib, the phospho-RTK array identified KIT, a target of sunitinib, as active in their tumors.[22] Similarly, the two patients with progressive metastatic alveolar soft part sarcoma who showed partial responses to sunitinib had active PDGFR on the array.[23] These findings implied that the phospho-RTK array might be useful as a diagnostic to predict individual tumor response to TKI therapy. In this report, we demonstrated that the phospho-RTK array can inform the TKI response profile of head and neck cancer cell line and patient derived xenograft (PDX) model.

Material and Methods

Cell lines, reagents and antibodies

The HNSCC cell lines (SCC9, SCC15, CAL27, SCC25 and MDA1386) were obtained, characterized, grown in media and condition as previously described.[21] All of the cell lines have been authenticated by short tandem repeat profiling within six months of passage. The phospho-receptor tyrosine kinase array was purchased (ARY001B, R&D Systems, Minneapolis, MN). The array layout of the 49 RTKs were shown in the Supporting Information Figure S5. The following small molecular TKIs were purchased (Selleck Chemicals): (*i*) JNJ-38877605, a highly selective, ATP-competitive inhibitor of c-MET[24]; (*ii*) NVP-AEW541, a potent inhibitor of IGF-1R with IC_{50} of 86 nM[25]; (*iii*) OSI-744/erlotinib HCl, a FDA approved EGFR inhibitor and (*iv*) STI-571/imatinib, a multi-target inhibitor of v-Abl, c-Kit and PDGFR. The TKIs were reconstituted in DMSO solvent as per manufacture recommendation.

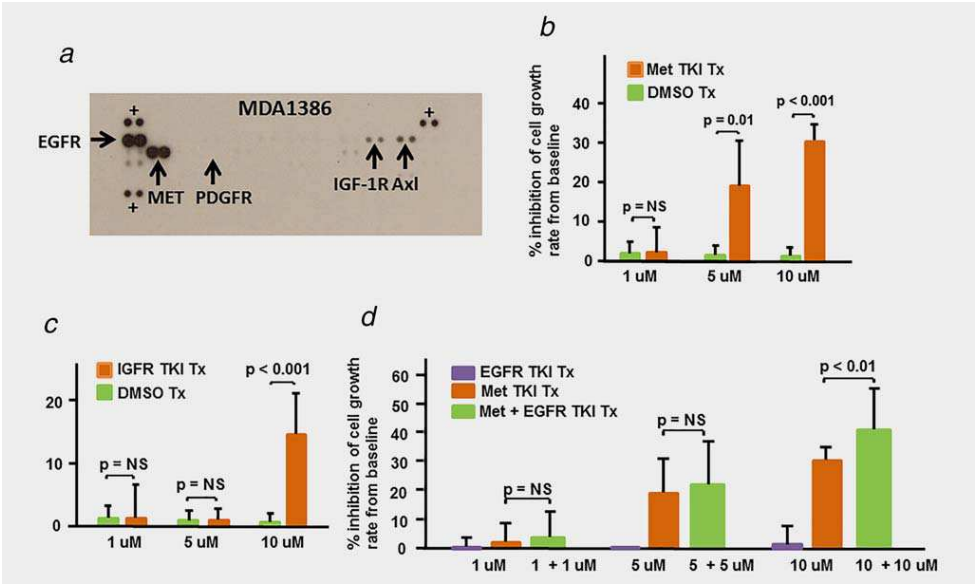


Figure 1. MDA1386 TKI response profile. (*a*) Phospho-RTK array analysis of MDA1386 cell lysate. The positive (+) controls are the built–in reference spots at the three corners of the array blot. Black arrows pointed to the positions corresponding to the respective RTK on the blot. Noted the strong EGFR and MET phosphorylation signals, the weaker IGF-1R and Axl signals and the absent PDGFR signal. (*b*) MDA1386 cell response to the MET TKI at three different concentrations (1, 5 and 10 uM, $n = 3$ at each concentration for each treatment). Tx: treatment; NS: not significant. Error bars represent ±2 standard errors. (*c*) MDA1386 cell response to the IGF-1R TKI at three different concentrations (1, 5 and 10 uM, $n = 3$ at each concentration for each treatment). Tx: treatment; NS: not significant. Error bars represent ±2 standard errors. (*d*) MDA1386 cell response to the dual inhibition of MET and EGFR in comparison to MET or EGFR inhibition alone at three different concentrations (1, 5 and 10 uM, $n = 3$ at each concentration for each treatment). Noted the increase in cell growth inhibition with dual MET and EGFR TKI at 10 uM. Tx: treatment; NS: not significant. Error bars represent ±2 standard errors. [Color figure can be viewed at wileyonlinelibrary.com]

Collection and processing of primary HNSCC tumors

Snap frozen primary HNSCC were collected through the Cooperative Human Tissue Network and the HNSCC tumor lysates were prepared for biochemical analyses by the homogenization method as previously described.[15,21] The snap frozen primary HNSCC were accrued as de-identified samples with no link to clinical information. On the other hand, the human HNSCC that used to establish the PDX were clinically annotated. All patients included in this study had given written informed consent. The collection of patients' materials for the PDX experiments and for the biochemical analyses was approved by the local Institutional Review Board of Charité University Medicine, Germany (EA4/019/12) and of the Stony Brook University respectively.

XTT proliferation assay

XTT proliferation assays were performed as previously described.[21] Briefly, cells were seeded at 10^4 cells/well in a 96 well plate in quintuplicate. The cells were treated the next day with increasing concentration of the corresponding tyrosine kinase inhibitor (1–10 uM) or DMSO control. Activated-XTT reagent was prepared and added to the cells the following day as per protocol. Cell proliferation rates were determined as previously described.[21] The proliferation rate of untreated cells served as baseline for comparison to that of treated cells. The percent of cell growth inhibition equaled one minus the proliferation rate of treated cells divided by that of untreated cells. A minimum of three independent experiments were performed at each concentration of treatment.

PDX treatment study and correlation analyses

Fresh tumor materials from patients who consented to the PDX treatment study was subcutaneously transplanted into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice as previously described.[26] Groups of 5–6 animals were randomized to treatment with cetuximab or saline as control according to schedule as described.[26] Tumor measurement was done at two dimensions with a sliding caliper twice a week during the three-week period of treatment. Treatment was initiated at a tumor size of 100–150 mm^3. Therefore, the experiments were performed as regression studies

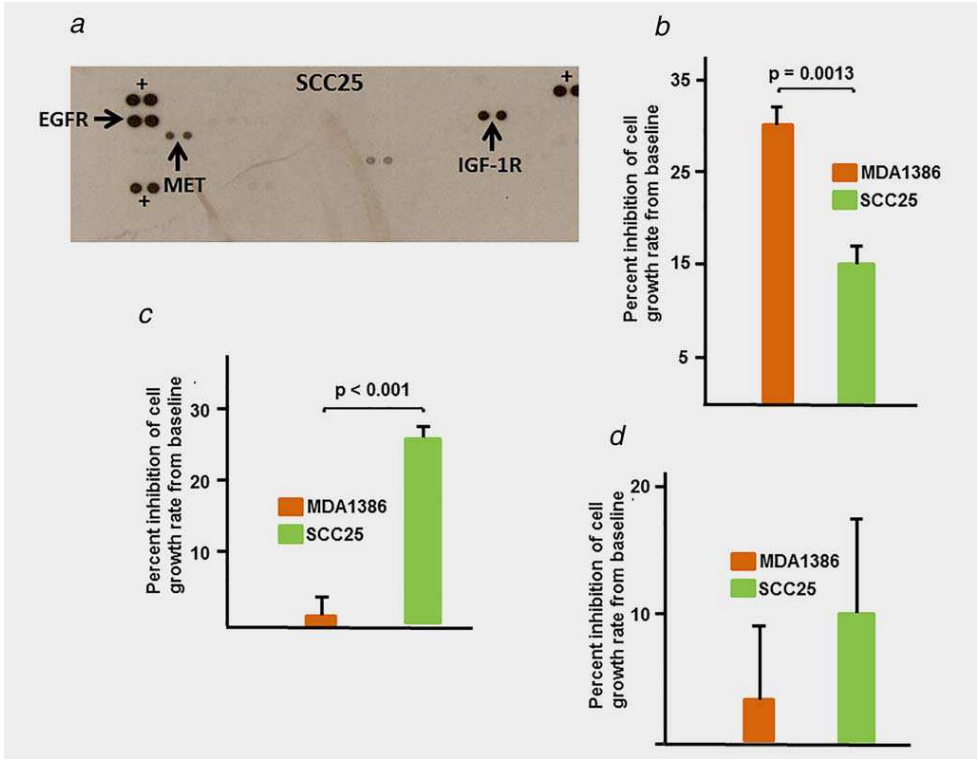


Figure 2. SCC25 cell line response to TKI. (*a*) Phospho-RTK array analysis of SCC25 cell lysate. Noted the strong EGFR and IGF-1R phosphorylation signals and the weaker MET signal. (*b*) Comparison of the degree of cell growth inhibition from baseline by MET TKI at 10 uM between the two cell lines (MDA1386 and SCC25, $n = 3$). Error bars represent ± 2 standard errors. (*c*) Comparison of the degree of cell growth inhibition from baseline by EGFR TKI at 10 uM between MDA1386 and SCC25, $n = 3$. Error bars represent ± 2 standard errors. (*d*) Comparison of the degree of cell growth inhibition from baseline by IGF-1R TKI at 5 uM between MDA1386 and SCC25, $n = 3$. Error bars represent ± 2 standard errors. [Color figure can be viewed at wileyonlinelibrary.com]

resembling the clinical situation. Individual tumor volumes (V) were calculated by the formula: $V = ([\text{width}]^2 \star \text{length})/2$. Change in tumor volume during the course of treatment was defined as the expression value most comparable to clinical tumor evaluation. Treatment response was defined by the relative tumor volume (RTV) which equaled tumor volume at the end of cetuximab treatment divided by that at the beginning of treatment. RTV of 0 is complete response (CR); RTV below 0.8 is partial response (PR); RTV between 0.8–1.2 is stable disease (SD) and RTV above 1.2 is progressive disease (PD). For the progressive tumor, the growth curve was examined and compared to the saline control. Primary resistance is defined as tumor growth curve overlapping the control curve, while secondary resistance is defined as increased tumor growth velocity at a later time point after an initial PR or SD. The different types of treatment response were illustrated in Supporting Information Figure S1. The investigators (E.L.C. and J.K.) who performed the phosphoarray were blinded to the cetuximab treatment response until the array results were analyzed. All animal experiments were carried out in accordance with the United Kingdom coordinating committee on cancer research regulations for the welfare of animals and the German Animal Protection Law, and the protocols were also approved by the local responsible authorities (LaGeSoBerlin, A0452/08).

Biochemical analysis

Cell, PDX and primary tumor lysates were analyzed for receptor tyrosine kinase signaling using the phospho-RTK arrays as per manufacture recommendation (R&D Systems). The following guidelines were established for the interpretation of the array result: (*i*) positive hit of a target (+) is defined as signal intensity stronger than or equally as strong as the positive controls on the same array blot; (*ii*) signals that are visibly about

Table 1. Summary of HNSCC PDX RTK phosphorylation profile and response to cetuximab

HNSCC PDX	pEGFR	pErbB2	Cetuximab response
11143	+	+	PR
10883	+	−	PR
11204A	+	−	SD
11857B	+	−	CR
11841	+	−	2^{o} resistance
11527A	−	−	1^{o} resistance
12346	+	−	SD
11873	+	−	SD
9876	+	+	1^{o} resistance
9897	+	+	1^{o} resistance
10114	+	+	CR
10309	+	+	1^{o} resistance
10321	+	+	1^{o} resistance
10621	+	+	1^{o} resistance
10913	+	+/−	SD
10924	+/−	−	1^{o} resistance
11303	+	−	1^{o} resistance
11452	+/−	−	1^{o} resistance
11857A	+/−	−	1^{o} resistance
13194	+	+	SD
11178	+	−	1^{o} resistance
11366	+	−	PR
10645	+	−	SD
11646	+	−	PR
12048	+	−	PR
11057	+	−	SD
11554	+	−	CR
11865	+	−	PR
11365	+	−	SD
11553	+	−	SD
11896	+	+	PR
11857	+	−	1^{o} resistance
11931	+	−	SD
11498	+	−	PR
11318	+	+	1^{o} resistance
10647	+	−	1^{o} resistance
10890	+	+/−	2^{o} resistance
11555	+	−	PR
11647	+	+	PR

half the intensity of the positive controls on the same array blot will be recorded as intermediate (±); and (*iii*) Signals that are less than half the intensity of the positive controls or nonvisible will be classified as negative (–). An illustration of the phosphoarray interpretation was shown in the Supporting Information Figures S2 and S4.

Statistics analysis

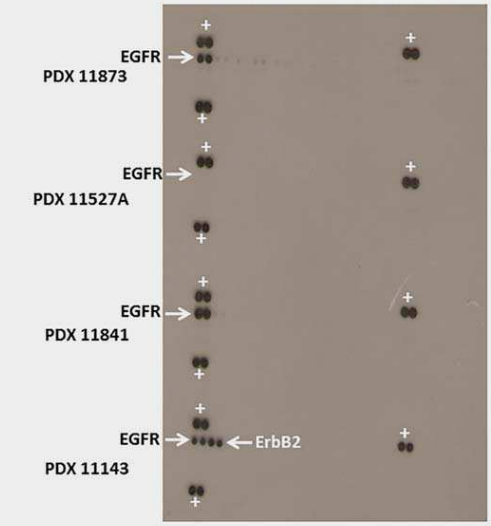
Statistical analyses were performed using SPSS Statistics 16.0 (SPSS, Chicago, IL). Comparison between the different drug treatment groups was performed using ANOVA. Fisher exact test was used to examine the association between pEGFR/pErbB2 status and cetuximab treatment response. Level of statistical significance is 5%.

Results

RTK phosphoarray predicts head and neck cancer cell response to kinase inhibitors

Using the phospho-RTK array, we determined that the HNSCC cell line, MDA1386, had activated EGFR and MET (Fig. 1*a*). We also noticed intermediate phosphorylation signals from IGF-1R and Axl, but no PDGFR activity in this cell line (Fig. 1*a*). Based on the array result, we treated the MDA1386 cells with the corresponding TKI. As expected, imatinib had no inhibitory effect on MDA1386 at all concentrations (Supporting Information Fig. S3). On the other hand, MET TKI treatment resulted in significant cell growth inhibition at 5 and 10 uM (Fig. 1*b*). While IGF-1R TKI also had a significant inhibitory effect on cell growth at 10 uM, it had no effect at 5 uM (Fig. 1*c*). This was predicted given the weak IGF-1R phosphorylation signal detected (Fig. 1*a*). Even though EGFR was highly phosphorylated (Fig. 1*a*), erlotinib had limited inhibitory effect on MDA1386 cell growth (Fig. 1*d*). This was also predicted because MET signaling is known to mediate EGFR TKI resistance.[27–31] To determine if dual EGFR and MET inhibition resulted in a greater therapeutic effect than MET TKI alone, we treated the MDA1386 cells with increasing concentrations of both inhibitors. As shown, the therapeutic effect of dual inhibition was significantly greater than that of either inhibitor alone (Fig. 1*d*). This implied a synergism between the two TKIs. In summary, the phospho-RTK array accurately informed the TKI response profile of the MDA1386 cell line.

Next, we determined the RTK phosphorylation profiles of four additional HNSCC cell lines. Surprisingly, all of the cell lines had similar profiles (Fig. 2*a* and Supporting Information Fig. S2). Since SCC25 had a weaker MET phosphorylation signal than MDA1386 (Figs. 1*a* and 2*a*), we decided to compare SCC25 response to MET TKI with that of MDA1386. While MET TKI inhibited the cell proliferation of both cell lines at 10 uM, its effect on MDA1386 was significantly stronger than that on SCC25 (Fig. 2*b*). We also tested SCC25 response to EGFR TKI. Based on the array result, we speculated that SCC25 would be more responsive to EGFR TKI than MDA1386 because MET activation was weaker in this cell line. Indeed, erlotinib resulted in a significantly greater growth inhibition in SCC25 than MDA1386 (Fig. 2*c*). On the other hand, we anticipated a greater effect of IGF-1R TKI on SCC25 than MDA1386 as SCC25 had a stronger IGF-1R phosphorylation signal. As anticipated, IGF-1R TKI resulted



b

	pEGFR+	pEGFR- or +/-
Cetuximab Response	20	0
Primary Resistance to Cetuximab	4	4

p = 0.003

c

	pErbB2+	pErbB2- or +/-
Cetuximab Response	5	20
Primary Resistance to Cetuximab	6	4

p = 0.04

Figure 3. RTK phosphorylation profile of HNSCC PDX. (*a*) Representative phospho-RTK array blots of four HNSCC PDX. Noted the strong EGFR and ErbB2 phosphorylation signals in PDX 11143 and the absent EGFR phosphorylation signal in PDX 11527A. (*b*) The association between pEGFR and cetuximab treatment response in the pErbB2 negative HNSCC PDX ($n = 28$). Cetuximab response included CR + PR + SD + the initially responsive tumor that later developed resistance (*i.e.*, secondary resistance). (*c*) The association between pErbB2 and cetuximab treatment response in the pEGFR+ HNSCC PDX ($n = 35$). Cetuximab response included CR + PR + SD + the initially responsive tumor that later developed resistance (*i.e.*, secondary resistance). [Color figure can be viewed at wileyonlinelibrary.com]

in a higher percentage of growth inhibition in SCC25 than MDA1386 (Fig. 2*d*). In summary, the phospho-RTK array informed the differential response of two HNSCC cell lines to kinase inhibitor therapies.

RTK phosphoarray predicts head and neck cancer PDX response to cetuximab

Using the phospho-RTK array, we determined the RTK phosphorylation profiles of 39 treatment naïve PDX tumors (Table 1). In contrast to the cell line data, none of the PDX had strong MET phosphorylation signal. While phosphorylated EGFR was the predominant signal in the majority (89.7%, 35/39) of PDX, 28.2% (11/39) had ErbB2 signaling (Fig. 3*a* and Table 1). We suspected that ErbB2 activation in the PDX is the result of EGFR-ErbB2 heterodimer signaling because (*i*) ErbB2 amplification rarely occurred in HNSCC[32] and no ErbB2 mutations were detected in any of the PDX[26]; (*ii*), all of the pErbB2$^+$ PDX ($n = 11$) had phosphorylated EGFR, but none of the pEGFR$^-$ PDX ($n = 4$) had pErbB2 (Table 1); (*iii*) ErbB2 receptor has no known ligand for its activation.[33] Based on these evidences, the only plausible explanation for ErbB2 activation in the PDX is EGFR-ErbB2 heterodimer signaling. Next, we correlated pEGFR signal with response to cetuximab treatment. Interestingly, there was a highly significant association between primary resistance to cetuximab and negative pEGFR signal in the pErbB2$^-$ PDX (Fig. 3*b*). The positive and negative predictive values of pEGFR status in predicting response in this PDX cohort were 83.3 and 100% respectively. Then, we examined the association between pErbB2 signal and response to cetuximab in the pEGFR$^+$ PDX. While the positive predictive value of a negative pErbB2 result in predicting cetuximab response in this PDX cohort was 83.3%, a positive pErbB2 signal did not necessarily predict cetuximab resistance (Fig. 3*c*). The association between pEGFR/pErbB2 signal and the clinical/pathological feature of each PDX was examined (Table 2). pErbB2 signal or the lack of pEGFR signal does not correlate to stage, tumor grade or location. Taken together, the phospho-RTK array informed the cetuximab treatment response in the HNSCC PDX.

The RTK phosphorylation profile of primary HNSCC is different from those of HNSCC PDX and cell lines

We were surprised to see the difference in RTK signaling pattern between the HNSCC cell lines and PDX. To determine

Table 2. Summary of HNSCC PDX clinical and pathological features. [Color table can be viewed at wileyonlinelibrary.com]

PDXID	TNM	Stage	Grading	Age	Site of tumor origin	Gender
9876	T3N2cM0	IVA	G3	62	Hypopharynx	Male
9897	T2N2bM0	IVA	G3	58	Hypopharynx	Male
1011*	T3N0M0	III	G3	52	Oral cavity	Male
10309	T4N2cM0	IVA	G3	55	Oropharynx	Male
10321	T2N0M0	II	G2	65	Oral cavity	Male
10621	T2N2bM0	IVA	G3	61	Oropharynx	Male
10645	T2N2CM0	IVA	G2	69	Oral cavity	Male
10647	T2N0M0	II	G2	65	Oral cavity	Male
10883	T4N0M0	IVA	G2	52	Oropharynx	Male
10890	T2N0M0	II	NA	NA	Oropharynx	Female
10913	T4N2ÒM0	IVA	G2	50	Oral cavity	Male
10924	T3N2CM0	IVA	G2	65	Hypopharynx	Male
11057	T1N0M0	I	G2	57	Oral cavity	Male
11143	T2N2ÒM0	IVA	NA	62	Oropharynx	Male
11178	T2N1M0	III	G3	60	Oral cavity	Male
11303	T3N1M0	IVA	G2	75	Oropharynx	Male
11318	T2N2bM0	IVA	G3	61	Oropharynx	Male
11365	T4bN2bM0	IVA	G2	59	Oral cavity	Female
11366	T2N0M0	II	G2	63	Oral cavity	Male
11452	T2N0M0	IVA	G2	75	Oral cavity	Male
11498	T2N2bM0	IVA	G2	67	Oropharynx	Male
11553	T4bN2bM0	IVA	G2	59	Oral cavity	Female
11554	T4N0M0	IVA	G2	68	Oral cavity	Female
11555	T4aN2bM0	IVA	G2	76	Oral cavity	Female
11646	T4aN2cM0	IVA	G2	71	Oral cavity	Male
11647	T4aN2cM0	IVA	G2	71	Oral cavity	Male
11841	T1N0M0	I	G2	56	Oral cavity	Female
11857	T4N2M0	IVA	G1	49	Oral cavity	Male
1186S	T4bN2cM0	IVA	G2	56	Oral cavity	Male
11873	T2N2MQ	IVA	G3	47	Oropharynx	Male
11836	T4bN2cM0	IVA	G2	56	Oral cavity	Male
11931	T2N2bM0	IVA	G2	61	Oral cavity	Male
17048	T2N2CM0	IVA	G3	46	Oral cavity	Male
12346	T1N2bM0	IVA	G3	76	Oropharynx	Male
13194	T4N2bM0	IVA	G2	50	Oral cavity	Male
11204A	T4N2cM0	IVA	G3	56	Oral cavity	Female
11527A	T2N2bM0	IVA	G2	74	Oral cavity	Male
11857A	T4N2M0	IVA	G1	49	Oral cavity	Male
11857B	T4N2M0	IVA	G1	49	Oral cavity	Male
pErbB2 +	pEGFR neg					

their similarity to the primary tumor, we performed phospho-RTK array analysis on nine freshly prepared primary HNSCC lysates. Unlike the cell lines, none had strong MET phosphorylation signal (Fig. 4 and Supporting Information Fig. S4). Like the HNSCC PDX, primary HNSCC had variable degree of EGFR phosphorylation (Fig. 4 and Supporting Information Fig. S4). This is consistent with our prior finding.[15] Nevertheless, the percentage of primary HNSCC with weak (±) to

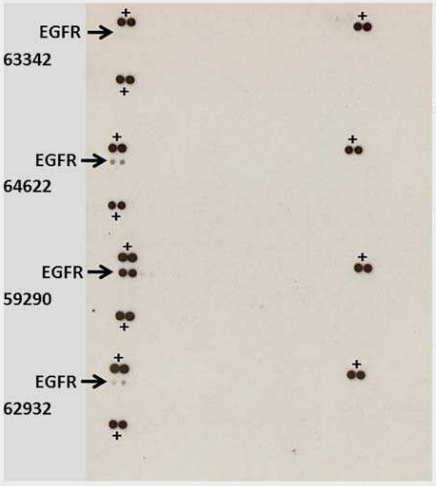


Figure 4. Representative phospho-RTK array blots of four primary HNSCC. Noted that only HNSCC 59290 had strong EGFR phosphorylation signal. [Color figure can be viewed at wileyonlinelibrary.com]

undetectable (–) phosphorylated EGFR (66.7%, 6/9) was higher than that of PDX (10.3%, 4/39). In addition, none of the primary HNSCC had ErbB2 activation (Fig. 4 and Supporting Information Fig. S4). Not detecting phosphorylated ErbB2/MET in the primary tumors suggested that the cell line or PDX might have acquired dependence on signaling pathways outside of the primary setting. Despite this subtle signaling difference between the preclinical model and the primary tumors, the phospho-RTK array is able to distinguish them and thus might also be useful in the clinical setting.

Discussion

Advancing precision medicine has become a national priority. Oncology is at the forefront of this initiative. The advance of genomic technology has made it possible to sequence tumor genome in real time to inform treatment decision and bring personalized medicine to cancer patients. While NGS is a promising platform, the limitation is its dependence on driver mutations as predictors of response to novel therapy. In this report, we tested the phosphoarray as a new platform for personalizing kinase inhibitor therapy. In both *in vitro* and *in vivo* model, the phospho-RTK array was able to inform the kinase inhibitor response of HNSCC cell lines and PDX. During the course of the study, we made several interesting observations. First, the result that 66.7% of primary HNSCC did not have detectable pEGFR signal is similar to our prior finding in a larger cohort using a different detection method (60.7%, 34/56).[15] This is in sharp contrast to the much lower percentage of HNSCC PDX with undetectable pEGFR signal (10.3%). Since negative pEGFR predicted primary cetuximab resistance in the PDX model, the high percentage of pEGFR$^-$ primary tumor might explain the low cetuximab response rate in HNSCC clinical trials.[34] Second, while the signaling profiles of HNSCC PDX bore close resemblance to that of the primary tumor, there were subtle differences. These differences might have been acquired during PDX passages. Thus, PDX response to targeted therapy should be carefully interpreted. Third, we noticed signaling and treatment response differences between PDX from different disease sites of the same patient (*i.e.*, 11857A: primary tumor *vs.* 11857B: metastatic site) and PDX from disease at different time point of the same patient (*i.e.*, 13194: primary tumor *vs.* 10913: recurrent tumor; Table 1). This finding supports assessing the molecular profile of not only tumors at different time points, but also tumors at different sites. Taken together, this study supports further development of the phosphoarray platform as a personalized diagnostic. Despite these promising results, there are several limitations with this platform. First, the TKI might not be potent enough to shut down the signaling of the target even when the right target was identified by the array. This could be secondary to a mutated target. Thus, the phosphoarray platform should be used in conjunction with the NGS platform to personalize TKI therapy. Second, there might be unknown compensatory mechanism(s) that conferred TKI resistance to the target identified. The phospho-RTK array does not include signaling pathways downstream of the RTK. Third, the array results can only be interpreted subjectively and do not take into account the differences in signaling strength. To improve on the currently available array, the next step will be to design and develop a quantitative array with expanded coverage of all potential druggable targets and resistant pathways. In conclusion, the phosphoarray technology and concept might be broadly applied to all cancer types and impact the field of personalized medicine.

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Molekulare Subtypen von Kopf-Hals Karzinomen

Klinghammer K, Otto R, Raguse JD, Albers AE, Tinhofer I, Fichtner I, et al. **Basal subtype is predictive for response to cetuximab treatment in patient-derived xenografts of squamous cell head and neck cancer**. Int J Cancer. 2017;141(6):1215-21

Molekulare Subtypen für Kopf-Hals Karzinome wurden in den vergangenen Jahren von verschiedenen Arbeitsgruppen publiziert. Die Einzelfaktoranalyse wie z.B. Amphiregulin oder phosphorylierter EGFR zeigte zwar eine Assoziiation zum Ansprechen bzw. Überleben, die notwendige Sensitivität und Spezifität zum Einsatz als prädiktiver Biomarker wurde damit jedoch nicht erreicht. In Anlehnung einer RNA basierten Klassifikation der Arbeitsgruppe von T.Seiwert (28) wurden für die Patienten-abgeleiteten Xenografts die molekularen Subtypen definiert, um durch die Analyse von Expressionsmustern eine Assoziation zu den verschiedenen getesteten Substanzen zu ermöglichen. In der Beschreibung der Subtypen zeigte sich ein basaler Typ mit einer hohen Aktivierung der EGF-Rezeptor Singalkaskade. Ein Nachweis, dass dieser Subtyp auch mit einer verbesserten Wirkung von Inhibitoren des Signalwegs assoziiert ist, konnte bislang jedoch nicht erbracht werden. Wir adressierten diese Fragestellung im PDX Modell und konnten zeigen, dass eine Wirksamkeit von Cetuximab im Basaltyp gegenüber dem Mesenchymaltyp signifikant häufiger ist. Für den dritten molekularen Subtyp, dem classical Typ bestand keine eindeutige Korrelation zur Wirkung von Cetuximab. In Einklang mit zuvor publizierten Daten zeigte sich, dass PDX Tumore vom Basaltyp gegenüber Mesenchymaltyptumoren eine deutlich erhöhte Singalwegaktivierung des EGFR Signalwegs aufwiesen. Hinsichtlich der Wirksamkeit klassischer Chemotherapien wie z.B. Cisplatin oder Docetaxel ergab sich kein Zusammenhang zwischen Wirkung im Tiermodell und molekularen Subtyp. Wir konnten damit erstmalig zeigen, dass ein Zusammenhang zwischen Signalwegaktivierung der EGF- Rezeptorsignalkaskade und der Wirksamkeit von Cetuximab besteht. Eine prospektive Evaluation der molekularen Subtypen als prädiktive Biomarker für den Einsatz von EGFR Antikörpern stand noch aus.

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Basal subtype is predictive for response to cetuximab treatment in patient-derived xenografts of squamous cell head and neck cancer

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Cetuximab is the single targeted therapy approved for the treatment of head and neck cancer (HNSCC). Predictive biomarkers have not been established and patient stratification based on molecular tumor profiles has not been possible. Since EGFR pathway activation is pronounced in basal subtype, we hypothesized this activation could be a predictive signature for an EGFR directed treatment. From our patient-derived xenograft platform of HNSCC, 28 models were subjected to Affymetrix gene expression studies on HG U133+ 2.0. Based on the expression of 821 genes, the subtype of each of the 28 models was determined by integrating gene expression profiles through centroid-clustering with previously published gene expression data by Keck *et al*. The models were treated in groups of 5–6 animals with docetaxel, cetuximab, everolimus, cis- or carboplatin and 5-fluorouracil. Response was evaluated by comparing tumor volume at treatment initiation and after 3 weeks of treatment (RTV). Tumors distributed over the 3 signature-defined subtypes: 5 mesenchymal/inflamed phenotype (MS), 15 basal type (BA), 8 classical type (CL). Cluster analysis revealed a strong correlation between response to cetuximab and the basal subtype. RTV MS 3.32 *vs*. BA 0.78 (MS *vs*. BA, unpaired *t*-test, *p* 0.0002). Cetuximab responders were distributed as following: 1/5 in MS, 5/8 in CL and 13/15 in the BA group. Activity of classical chemotherapies did not differ between the subtypes. In conclusion basal subtype was associated with response to EGFR directed therapy in head and neck squamous cell cancer patient-derived xenografts.

Introduction

Head and neck cancer comprises a heterogeneous group of tumors arising from the upper aero digestive tract. The most common histology of tumors arising in the oral cavity, oropharynx, hypopharynx and larynx is squamous cell carcinoma (HNSCC). Despite recent advances in the diagnosis and treatment, especially the metastatic and recurrent setting remains a major challenge with fatal outcome for most patients within a few months after diagnosis. Within the last years the molecular understanding of HNSCC has increased tremendously. Especially large multi-center, national or even international efforts, such as the cancer genome atlas project led to a deeper understanding of the heterogeneous landscape of molecular aberrations found within this disease.[1–3]

In addition, gene expression analyses have proven to be a useful tool for classification of tumors. For breast cancer such tools have arrived in the clinical routine for the evaluation of the risk of recurrence and thereby influence the decision for adjuvant chemotherapy.[4,5] Several groups have independently defined molecular subtypes of HNSCC based on gene expression profiles.[6–9] All groups defined a basal-like subtype which is characterized by high expression of epidermal growth factor receptor (EGFR) pathway members, and a classical subtype, which is defined by high expression of cell cycle genes and genes associated with xenobiotic exposure. Furthermore, mesenchymal and atypical subtypes have been defined, whereas

Key words: head and neck cancer, patient-derived xenograft, molecular subtype, cetuximab, EGFR
Additional Supporting Information may be found in the online version of this article.
K.K. and R.O. contributed equally to this work
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What's new?
Head and neck squamous cell carcinoma (HNSCC) has multiple subtypes, including basal and classical subtypes, which exhibit elevated expression of epidermal growth factor receptor (EGFR) pathway members. EGFR inhibition is the central action of cetuximab, the only targeted treatment approved for HNSCC. Whether EGFR expression predicts cetuximab response, however, is unclear. In our study, analysis of whole gene expression in patient-derived xenografts revealed a positive association between the basal subtype of HNSCC and cetuximab response. By contrast, mesenchymal subtype tumors were associated with cetuximab resistance. Functional analyses revealed an enrichment in EGFR pathway gene expression in the basal subtype.

some authors combined the latter two in one subgroup. The different molecular subtypes have been associated with a variety of clinical and biological characteristics such as histological appearance, lymph node metastasis, HPV status and patient survival.[6,8,10,11] Unfortunately, the gain of knowledge and understanding of the underlying biology in HNSCC has not led to defining novel biomarkers or changed clinical practice yet. Treatment decision in clinical routine remains based on conventional factors such as tumor stage, patient performance, comorbidities and patient wish.

To date, cetuximab, a monoclonal antibody directed against the extracellular domain of EGFR is the only approved targeted treatment for HNSCC. EGFR protein expression evaluated by immunohistochemistry and gene amplification was not found predictive for EGFR targeted treatment.[12,13] Currently, no clinically validated biomarker exists, which would allow treatment stratification. It has been questioned whether HPV-positive tumors respond to EGFR directed treatment not least because adequate preclinical models are scarce and translational studies on patient-derived material have the difficulty that patients are rarely treated with a single agent to define a particular biomarker next to the challenge of appropriate tissue availability.[14] Patient-derived xenografts (PDX) have been defined as preclinical models, reflecting patient tumors very closely. Therefore, they are accepted as suitable models for biomarker studies and compound evaluation.[15–17]

To further engage into biomarker research our panel of head and neck PDX was evaluated for whole gene expression as well as mutational patterns. Based on previous work,[6] we hypothesized that the basal subtype gene expression signature of HNSCC could be predictive for cetuximab response, due to the activation of the EGFR pathway axis.

Materials and Methods

PDX generation, treatment and evaluation of response

All patients included in our study had given written informed consent. The study was approved by the local Institutional Review Board of Charité University Medicine, Germany (EA4/019/12). All animal experiments were carried out in accordance with the United Kingdom coordinating committee on cancer research regulations for the welfare of animals and the German Animal Protection Law, and were also approved by the local responsible authorities (LaGeSoBerlin, A0452/08).

Fresh tumor material was subcutaneously transplanted into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice as described previously.[17] Each tumor model was assigned with a number, in case of synchronous establishment of PDX from the primary tumor and a lymph node metastasis from the same patient, this was indicated with an A or B, respectively. After histological tumor validation PDX were used for response evaluation to classical chemotherapies such as docetaxel, carbo- or cisplatin, 5-fluorouracil, everolimus and cetuximab within the 2nd and 4th passage. Dosage and route of administration are shown in Supplementary Table S1. Tumor measurement was done at two dimensions with a sliding caliper twice a week during the 3-week period of treatment. Treatment was initiated at a tumor size of 100–150 mm^3. Therefore, the experiments were performed as regression studies resembling the clinical situation. Individual tumor volumes (V) were calculated by the formula: $V = ([\text{width}]^2 * \text{length})/2$. Change in tumor volume during the course of treatment (RTV) was defined as the expression value most comparable to clinical tumor evaluation. In accordance to the Response Evaluation Criteria in Solid Tumors (RECIST) four groups of responders were defined: Complete remission, CR (RTV <0.2), partial remission, PR (RTV 0.2–0.7), stable disease, SD (RTV 0.8–1.2) and progressive disease, PD (RTV >1.2).

Patient survival analysis with regard to engraftment

Follow-up data from patients enclosed in the study were collected retrospectively. Excluded were patients whose tumors grew out as lymphoma in mice ($N = 22$) and patients with incomplete follow-up data ($N = 17$). Overall survival was defined as time from tumor resection to death or the date of the last contact. Engraftment in mice was stated, if stable growth of tumors was observed after 2nd passage Multivariate cox regression analysis was used to calculate established prognostic factors. For Cox regression and Kaplan–Meier survival analysis SPSS version 21 was used. Due to missing results from drug testing, two models were not eligible for analysis.

Mutational analysis and integration

Tumors grown as xenograft models were subjected to Next Generation Sequencing (NGS) analysis as described previously.[17]

Mutational load was evaluated by comparing the number of variant calls excluding annotated germline polymorphisms as indexed at dbSNP NCBI database. Mutations in *TP53* and *PI3KCA* genes were analyzed for enrichment in different molecular subtypes, since these genes were frequently altered in our PDX HNSCC panel.

mRNA expression analysis

Tumor RNA of PDX-derived tumors from the untreated control group was extracted from each sample using Qiagen RNeasy Kit according to the manufacturer's protocol. The integrity of RNA was determined using the Agilent 2100 Bioanalyzer. Total RNA were assayed using Affymetrix HG U133+ 2.0 microarrays evaluating >47,000 transcripts. Quality control procedures were applied to probe level intensity files. Raw data were deposited with accession number GSE84713 on GEO repository (http://www.ncbi.nlm.nih.gov/geo/).

The R-package "affyPLM"[18] was used to background-correct CEL files with the GC-RMA[19] algorithm. Initial quality analyses included PCA-clustering of raw-data, analysis of mRNA-degradation and MA-Plot outlier-detection. Next, expression data were quantile-normalized and the R-package "LIMMA"[20] was applied for linear differential expression analysis. Differential expression *p*-values and *Q*-values were defined significant when they were lower than 0.05. The *p*-values were corrected for multiple testing by applying a Benjamini and Hochberg[21] correction to obtain *Q*-values. The significant absolute log fold-change was set to one. For gene-set enrichment analysis, we used the Broad Institute R-Package "GSEA"[22] with MSigDB[23] signature file "c2.all.v5.1.symbols.gmt."

Centroid cluster analysis

Sample subtypes were determined by identifying the reference subtype-clusters to which they were most significantly similar, where reference-clusters of the subtypes were represented as their centroids, i.e. scaled characteristic subsets of genes. A sample was defined as being significantly similar to a subtype-centroid if the *Q*-value of the Pearson-correlation *p*-values was lower than 0.05, as shown in Supplementary Table S4. The reference-centroids were obtained from Keck *et al.*[10] and De Cecco *et al.*[7] To account for differences in the exact definition of the clusters in those works, we performed cluster analyses four times, each time, each on a different subset of genes: 821 genes (following[10]), 2,843 genes (following[7]), 756 (Keck *et al.* genes minus 65 genes reported to be mouse stroma-associated[24]) and finally the 300 strongest subtype defining genes. In case several probes mapped to the same gene, only the probe with the highest variance was used. The use of the algorithm developed by De Cecco *et al.* for centroid clustering of our PDX expression dataset did not result in distinct subtyping of 50% of our samples.[7] All analyses were run with the Statistics Software R, version 3.2.

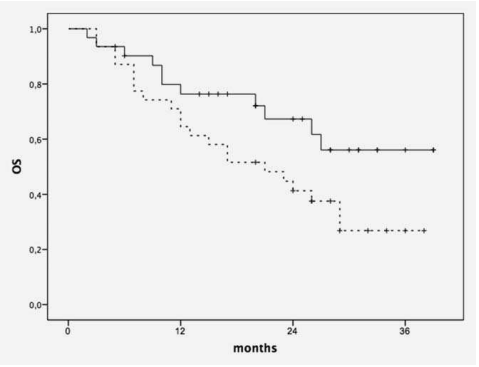

Figure 1. Kaplan–Meier survival curve: dotted line resembles patient survival whose tumor engrafted in PDX, continuous line resembles patients whose tumor did not grow on mice. Log rank (Mantel–Cox) $p = 0.053$. The average survival of patients whose PDX engrafted was 21.1 months in comparison to no engraftment with 28.4 months

Results

PDX platform

As described previously, we generated a large HNSCC xenograft platform for compound evaluation and biomarker research. By collecting follow-up data for 62 patients, who were diagnosed and treated at the Charité University hospital and enclosed in the study, we here investigated the prognostic value of the tumor engraftment. Patient characteristics are shown in Supplementary Table S2. Groups were balanced in regard to engraftment: $n = 30$ engrafted and $n = 32$ no engrafted. Survival analysis revealed a trend to a higher probability of death, if tumors did growth on mice with a hazard ratio of 2.007, (95% CI: 0.967–4.165); Rank (Mantel–Cox) $p = 0.053$ (Fig. 1); furthermore, average overall survival for patients whose tumors engrafted was 21.1 months (95% CI: 16.6–25.7) whereas patients whose tumor did not grow on mice had an average overall survival of 28.4 (95% CI: 23.4–33.4) months. Interestingly, traditional prognostic factors of HNSCC such as tumor stage, differentiation, gender, age and site of tumor origin did not influence overall survival in our cohort.

Gene expression – class definition

Twenty-eight PDX of head and neck cancer were evaluated for whole gene expression (detailed clinical information of the 28 models are summarized in Supplementary Table S3). Applying the subtype definition described by Keck *et al.* we were able to classify the 28 PDX models by gene expression values of 821 genes into the three major groups of basal (BA $n = 15$), classical (CL $n = 8$) and mesenchymal/inflamed (MS $n = 5$) subtype. Most samples clustered in the basal subtype. The two HPV-positive models HN10309 and HN11303 were assigned to the classical and mesenchymal/inflamed subtype, respectively.

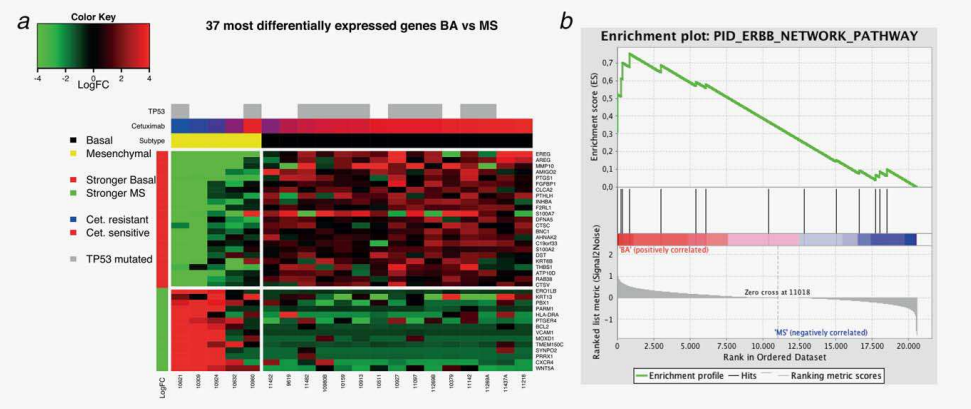

Figure 2. (*a*) Heatmap of 37 genes with greatest differential log-fold change between basal and mesenchymal subtypes. Numbers on the *x*-axis stand for PDX models. The R-package's LIMMA linear model differential expression algorithm was utilized and the LogFC threshold set to 2 and the *p*-values to 0.05. The underlying differential expression analysis showed 757 differentially expressed genes between the basal and mesenchymal subtypes of which 37 with a LogFC of at least 3.8 were chosen for visualization. (*b*) Functional analysis enrichment plot of ERBB network pathway displaying a positive correlation of basal subtype *vs*. a negative correlation of mesenchymal subtype. Employing Benjamini–Hochberg Multiple-Testing Correction the FDR had a *p*-values 0.003 and *Q*-value 0.023 [Color figure can be viewed at wileyonlinelibrary.com]

Since stroma associated genes expression change during the process of tumor engraftment in mice, we explored whether stroma associated genes influenced the class definition. We used results from Peng *et al.* who previously defined differently expressed genes between head and neck patient tumors and thereof derived PDX.[24] Sixty-five of these differently expressed genes between patient tumors and PDX were found in the set of 821, which defined the molecular subtype. In a second analysis, we repeated molecular subtyping after removing these 65 genes, which did not result in change of subtype assignment for any of the samples.

For visualization, a cluster heatmap was created showing the PDX samples' clustering-pattern based on their pair-wise correlation of the expression of the 821 signature-defining genes from Keck *et al.* Utilization of the expression of the 821-genes during the single-linkage clustering did not create a clustering-pattern that corresponded classification-subtypes although all *p*-values were significant. Causes may be due to low sample number in conjunction with the fact that the signature was trained on different samples and different technologies, thereby causing a low signature-to-signal ratio (Supplementary Fig. S1*a*). We therefore increased the noise-to-signal ratio of the data by restricting the analysis to the 300 strongest class-defining genes i.e. above-noise genes with the result of a clear distinction of the three groups (Supplementary Fig. S1*b*). When re-classifying samples based on the 300 gene-signature, only a single sample (HN11143) changed its subtype from classical to mesenchymal, the remaining classifications stayed identical to the 821 gene-signature.

Functional analysis

We further evaluated functional pathway enrichment employing gene set enrichment analysis (GSEA) between the three major subtypes. As expected, the EGFR pathway was positively enriched within the basal subtype (enrichment score 0.75, $p = 0.01$). Gene expression values differed between the basal and mesenchymal subtypes significantly especially for *EREG*, *AREG*, *EGFR*, *NRG1* and *HBEGF*.

Furthermore, the enrichment plot revealed a positive correlation of well-differentiated tumors with a basal subtype (enrichment score 0.72, $p = 0.02$) in comparison to mesenchymal subtype which showed a negative enrichment as shown in Figures 2 and 3.

Correlation of subtypes with response to treatment in head and neck PDX

All 28 PDX models with complete datasets for response to single-agent treatment with either docetaxel, carbo- or cisplatin, 5-fluorouracil, cetuximab or everolimus were evaluated for correlation of the subtype with the response to treatment. Whereas responses to docetaxel, platinum compounds, everolimus or 5-fluorouracil were not enriched in any particular subtype as shown in Supplementary Figure S2. However, there was a significant enrichment of cetuximab responders within the basal subtype. In contrast, models classified as mesenchymal/inflamed subtype were less likely to respond to cetuximab (unpaired *t*-test BA *vs*. MS $p = 0.0002$) as shown in Figure 4.

Mutational profiles

We further analyzed whether common mutations in head and neck cancer can be found predominantly in one of the

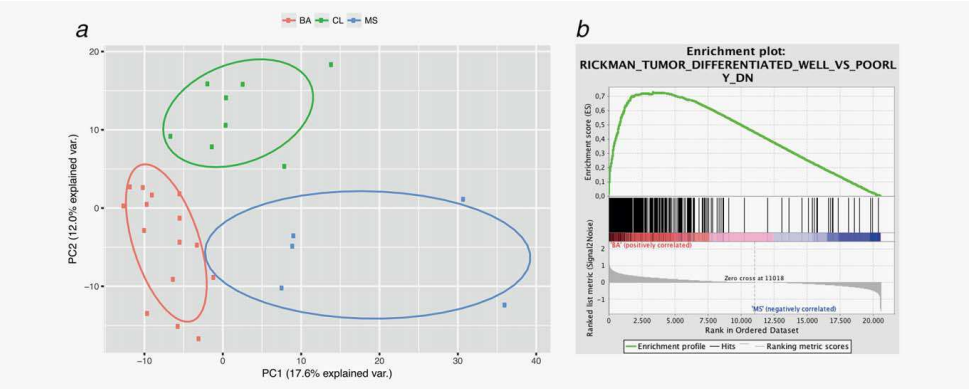

Figure 3. (*a*) Principle component analysis of the 3 subgroups based on the 821 subtype-defining genes by Keck *et al.*: BA basal, CL classical and MS mesenchymal. Only genes of the 821-gene signature were used after filtering the 10% least expressed genes. Mesenchymal subtype is negatively correlated with tissue differentiation, (*b*) in comparison to basal subtype, which is positively correlated [Color figure can be viewed at wileyonlinelibrary.com]

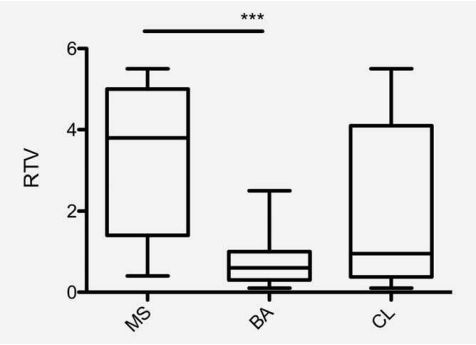

Figure 4. Cetuximab response expressed as relative tumor volume (RTV) in different subtypes of head and neck cancer. Mesenchymal subtype (MS $n = 5$), mean RTV 3.3, basal subtype (BA $n = 15$), mean RTV 0.78 and classical subtype (CL $n = 8$), mean RTV 2; BA *vs.* MS $p = 0.0002$ indicated by ***

subgroups and whether the mutational profile was correlated with response in xenograft models. The median number of mutations identified by panel sequencing (Illumina Cancer panel) of our models was 42,6 per sample. Within the different subtypes no significant differences were observed with regard to mutational load (BA 42.7 *vs.* CL 44.5 *vs.* MS 40). *TP53* and *PI3KCA* were the most frequently altered genes found in our cohort. While the frequency of *TP53* mutation was equally distributed across different subtypes, we observed a clear enrichment of *PI3KCA* mutation in MS subtypes. The percentages of mutated *PI3KCA* samples within the subgroups were BA 13.3% *vs.* CL 16.6% *vs.* MS 60%. However, no association of response and mutational status of *TP53* or *PI3KCA* to any of the evaluated compounds was observed.

Discussion

Cetuximab is the only targeted agent approved for the treatment of head and neck cancer and so far no predictive biomarker has been identified and clinically validated. In our study, we reported a significant association between the response to cetuximab and the molecular subtype evaluated by gene expression in HNSCC PDX. By assigning molecular subtypes in 28 HNSCC PDX, we were able to show that basal subtype was associated with response to cetuximab. In contrast, the mesenchymal subtype was associated neither with growth delay nor with tumor regression when treated with cetuximab. Bossi *et al.* recently showed that patients with a long progression-free survival under cetuximab maintenance therapy can be predominantly found in the basal subtype group.[25] This is very much in line with our findings and supports the notion of prospective evaluation of gene expression profiles in regard to patient stratification. Based on our preclinical data the positive and negative predictive value of basal subtype to predict cetuximab response was 68% and 77%, respectively, whereas the negative predictive value for mesenchymal subtype reached 94%.

Chung *et al.* were the first that proposed four molecular subtypes evaluating 60 head and neck tumor gene expression profiles and showed that patients within the group with EGFR activation had the worst prognosis.[6] Therefore, the hypothesis that tumors with EGFR activation might derive a major benefit from EGFR antibody treatment was raised but remained a major challenge to prove, since the group also showed that available cell lines for preclinical evaluation did

not cluster within patient expression profiles. Furthermore, in the clinical setting patients were usually not treated with single agent cetuximab rather than combined treatment.

Within our work we reported functional pathway analysis that revealed a high expression of genes found in the EGFR pathway especially in the basal subtype. Gene expression of *AREG* and *EREG*, which are ligands to the EGFR, have previously been associated with cetuximab response in colorectal cancer.[26]

Beside the clustering analysis algorithm developed by Keck *et al.* which we employed for our work, De Cecco *et al.* independently also suggested a subtype clustering model for head and neck cancer.[7,10] For comparative analysis, we evaluated the distribution of our PDX models over the six subtypes defined by De Cecco and co-workers. This did not result in a significant mapping for almost 50% of our PDX models. We therefore restricted our analysis to the classification developed by Keck *et al.* However, both groups defined a classical subtype with similar phenotype. By evaluating cell line response data for rapamycin De Cecco hypothesized the classical subtype (Cl5) might respond to mTOR inhibitor treatment. We could not confirm this hypothesis in our PDX models: Tumors responding to treatment with the mTOR inhibitor everolimus and non-responders did not cluster to different subtypes.

Next to histological appearance, genetic profile and protein expression it has been shown by several groups that gene expression patterns of patient tumors are mirrored in PDX.[15,27–29] Therefore, PDX are considered as models that resemble the primary tumors. However, some restrictions remain: Especially tumors with a clinically aggressive behavior resulted in stable growth in mice. So far no predictive marker of successful engraftment in mice has been identified for HNSCC. By following up patient survival, we were able to show that successful tumor engraftment in mice is associated with a poorer prognosis. Traditional prognostic factors for patients with head and neck cancer such as TNM were not mirrored in our cohort, which might be due to tumor selection at operation toward large, high T-stage carcinomas during the process of establishment. We therefore have to be aware of selecting for this unfavorable yet clinically important patient cohort and our findings might not be applicable to all patients.

Furthermore, within this work we evaluated only a limited number of PDX. Taking into consideration the differences of PDX and human tumors the findings reported are indicative and cannot be applied to patients without further evaluation in a clinical setting. However, with the previous reports from other groups, our work adds further evidence of the predictive value of gene expression signature for cetuximab treatment, creating a rationale for evaluation in a patient cohort. In addition, future research regarding the functional differences of the subtypes is motivated by the findings of this work.

Acknowledgement

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Diskussion

Personalisierte Krebsmedizin ist der Wunsch eines jeden Patienten, Onkologen und nicht zuletzt des Kostenträgers. Bei genauer Betrachtung sind wir heute nur bei einer Handvoll von Krebserkrankungen in der Lage zielgerichtete Medikamente auszuwählen. Beispiele dafür sind Trastuzumab beim Her-2 überexprimierenden Mammakarzinom oder Imatinib beim Vorliegen des bcr-abl Fusionstranskripts bei der CML. Eine Therapie dem Tumor bzw. den genetischen Eigenschaften des Patienten anzupassen, ist gegenüber der Chemotherapie in der Regel mit einer besseren Verträglichkeit und höherer Wirksamkeit assoziiert. Für viele Medikamente fehlen in der täglichen Routine jedoch validierte Testverfahren, die eine entsprechende Selektion ermöglichen. Aufgrund dieses Mangels werden Medikamente eingesetzt ohne vorab zu wissen, ob ein Patient tatsächlich von der Behandlung profitieren wird oder nur die Toxizitäten erlebt. Darüber hinaus entstehen insbesondere für neuere Medikamente zum Teil enorme Kosten, die durch eine adäquate Patienten- bzw. Medikamentenselektion gesenkt werden könnten.

Die dargestellten Arbeiten haben sich insbesondere mit der Definition prädiktiver Biomarker zum Einsatz von Cetuximab befasst, da bis zum heutigen Zeitpunkt kein einziger validierter Biomarker in der Behandlung der Kopf-Hals Karzinome zur Verfügung steht.

Durch die Entwicklung immundefizienter Mäuse ist es gelungen, die Anwachsraten von humanen Tumoren in diesen Tieren zu verbessern und dadurch für wissenschaftliche Fragestellungen nutzbar zu machen. Dieses Avatarmodell wird als Patienten-abgeleitetes Xenograftmodell (PDX) bezeichnet. Mittlerweile gibt es annähernd jede Tumorart als PDX.(29) Bemerkenswert ist die histopathologische Ähnlichkeit der PDX verglichen mit dem Ausgangstumor, die Entitäten übergreifend von verschiedenen Arbeitsgruppen gezeigt werden konnte.(30-32) Relevant für den Stellenwert der präklinischen, translationalen Forschung im Tiermodell ist letztlich wie gut sich die Ergebnisse auf die Patientensituation übertragen lassen. In der Vergangenheit sind viele Erkenntnisse in der Übertragung ans Krankenbett gescheitert, aufgrund der geringen Repräsentanz von Zelllininen gegenüber dem Ausgangstumor.(33) Eine wesentliche Verbesserung der PDX gegenüber Zelllinien ist

der Erhalt eines Tumorstromas, das allerdings aus murinen Komponenten besteht. Dadurch ist die Evaluation von Substanzen, die in den *crosstalk* von Tumorzellen und Stroma eingreifen in PDX prinzipiell möglich.(34) Weiterhin bilden die Tumore in den PDX ihre eigenen Blutgefäße, so dass Substanzen, die in die Angiogenese eingreifen in diesen Modellen untersucht werden können.(35) Die Frage, inwieweit Mutationsprofile in PDX erhalten bleiben, wurde von uns sowie vielen anderen Arbeitsgruppen untersucht. Zusammenfassend zeigte sich ein hohes Maß der Korrelation der Mutationsprofile zwischen Ausgangstumor im Patienten und PDX (Korrelationskoeffizient R=0.94). Im Unterschied dazu konnte diese Beobachtung in Zelllinien nicht gemacht werden, mit einem geringerem Korrelationskoeffizient von R=0.51.(36) Neben Mutationsprofilen wurden Genexpressionsprofile von Primärtumoren und davon abgeleiteten Xenografts verglichen. Unsupervised hierarchical clusting zeigte wiederholt die enge Beziehung zwischen Ausgangstumoren und den daraus abgeleiteten Xenografts.(37, 38) Zusammenfassend ergibt sich, dass die biologischen Eigenschaften eines humanen Tumors weitestgehend im PDX erhalten bleiben. Limitationen im Umgang mit dem Modell gibt es aber dennoch. Zum einen wachsen nicht alle transplantierten Tumore auf den PDX. Die Anwachsraten variieren zwischen 20-80% je nach Arbeitsgruppe und Tumortyp. Ein klarer Prädiktor für Engraftment konnte bislang nicht identifiziert werden, wobei insbesondere undifferenzierte Tumore, die ein aggressives Wachstumsverhalten zeigen, besser im Tiermodell anwachsen. Eine weitere Limitation ist das Fehlen eines intakten Immunsystems in der Maus. Insbesondere neuere, in der Behandlung von Tumorerkrankungen eingesetzte Immuntherapeutika, können dadurch nicht adäquat in diesem Modell untersucht werden. Eine andere Hürde stellen hohe Kosten dar, die durch die Tierhaltung und die notwendige Infrastruktur vorgehalten werden müssen. Bis zum Anwachsen eines Tumors vergehen durchschnittlich 70 Tage. Der Einsatz von PDX als Vorhersagemodell für das Therapieansprechen eines Patienten ist in der klinischen Routine bis heute nicht durchführbar. PDX sind dennoch wertvolle Modelle, weil durch die Kenntnis der Tumorbiologie des einzelnen Tumors besonders gut Modelle für die Testung spezifischer Substanzen selektiert werden können. Durch die große Zahl unterschiedlicher Modelle einer Tumorentität kann eine Phase II Studie simuliert (sog. Mouse clinical trials) und damit als Indikator für die weitere klinische Entwicklung verwendet werden.

Positiv und negativ prädiktive Biomarker für den Einsatz von Cetuximab: EGFR Expression

Die Expression der Zielstruktur ist hinsichtlich der Wirksamkeit für die Mehrzahl der Substanzen relevant. Beim Her-2 Rezeptor konnte gezeigt werden, dass Trastuzumab nur dann einen klinisch relevanten Nutzen zeigt, wenn eine Amplifikation des Rezeptors nachgewiesen werden kann. 90-100% der plattenepithelialen Kopf-Hals Karzinome exprimieren den EGFR und eine Überexpression wurde als unabhängiger prognostischer Marker identifiziert, der mit grossen Tumoren, einer verminderten Radiosensitivität und einem hohen Rezidivrisiko assoziiert ist.(39-41) Der Hauptmechanismus, der zu einer Überexpression führt, besteht in einer Amplifikation des EGFR Genabschnitts.(42) Weder für das kolorektale Karzinom noch für Kopf-Hals Karzinome konnte gezeigt werden, dass die Expression ob als Protein oder auf Ebene der RNA relevant für das Ansprechen auf Cetuximab ist.(43) In unseren Expressionsstudien konnten wir ebenfalls keinen Zusammenhang zwischen der Wirksamkeit des Antikörpers und der Proteinexpression belegen. Im Rahmen der translationalen Forschung am Kollektiv der EXTREME Studie erfolgte die Untersuchung inwieweit eine Genamplifikation prädiktiv ist. Auch in diesen Studien zeigte sich keine Assoziation.(44)

Zusammenfassend ergibt sich aus einer Vielzahl von Untersuchungen in unterschiedlichen Krankheitsentitäten kein Zusammenhang zwischen der Expression von EGFR und der Wirksamkeit von Cetuximab. Im präklinischen PDX Modell zeigte sich allerdings, dass die Phosphorylierung des EGFR als Ausdruck der Aktivierung des Rezeptors assoziiert war mit Cetuximab Response. Diese Ergebnisse bedürfen noch einer Validierung in einer Patientenkohorte.

Während Tyrosinkinasemutationen des EGF-Rezeptors beim Adenokarzinom der Lunge vorkommen und relevant für die Therapieselektion sind, treten diese bei Plattenepithelkarzinomen aus dem Kopf-Hals Bereich nicht gehäuft auf. Eine molekulare Alteration, die initial mit einer Frequenz von bis zu 42% berichtet wurde, ist die trunkierte EGFRvIII. Dabei kommt es zur Deletion der Aminosäuren 6-273 entsprechend der extrazellulären Domäne des EGFR mit der Folge einer konstitutiven Aktivierung.(45, 46) Die transmembranäre und intrazelluläre Domäne des Rezeptors unterscheiden sich nicht vom Wildtyp. Funktionell besteht eine dauerhafte Phosphorylierung und dadurch Aktivierung, obwohl keine extrazelluläre Bindungsdomäne mehr vorhanden ist und eine Bindung der Liganden nicht mehr

möglich ist.(47) Interessanterweise erfolgt die Signalübertragung ausschließlich über den PI3K Signalweg und nicht wie beim EGFRwt üblicherweise über Ras-Raf-MAPK.(48) Wir untersuchten die Rolle von EGFRvIII in einem Kollektiv von Kopf-Hals Tumorpatienten, die mit Docetaxel und Cetuximab behandelt wurden. Wir konnten eine deutlich verringerte Erkrankungskontrolle (13%) bei Vorliegen einer hohen EGFRvIII Expression gegenüber einer niedrigen Expression (65%) belegen. Der Nachweis erfolgte mittels immunhistochemischer Färbungen. Für die klinische Anwendung als negativ prädiktiver Marker zur Selektion von Patienten, die von einer EGFR gerichteten Therapie mutmaßlich nicht profitieren, fehlt bis heute ein validierter Antikörper. Darüber hinaus eignen sich alternative Nachweisverfahren wie z.B. RNA Sequenzierung bislang nicht für den breiten klinischen Einsatz. Die Ergebnisse der Sequenzierungsstudien (TCGA), in denen kein Tumor mit einer vIII des EGFR detektiert wurde, haben die Frage nach der tatsächlichen Häufigkeit aufgeworfen, was bislang nicht abschliessend geklärt wurde.(49)

Single Nucleotid Polymorphismen des EGFR

Polymorphismen sind genetische Varianten, die in der Allgemeinbevölkerung natürlicherweise vorkommen. Ein Polymorphismus im EGFR in der Ligandenbindungsdomäne bezeichnet den R521K, vormals Her497. Dieser wurde 1994 erstmals beschrieben und funktionell charakterisiert. Es zeigte sich, dass beim Vorliegen der Variante nach Stimulation mit den Liganden TGF-alpha oder EGF eine geringere Signalwegaktivierung erfolgte als im Vergleich zur Wildtypvariante.(50) Weiterhin wurde die prognostiche Relevanz des Polymorphismus in einem Kollektiv von Patienten mit kolorektalen Karzinomen untersucht. In dieser Kohorte von mehr als 200 Patienten zeigte sich eine geringe Aktivierung von c-Myc, geringere Phosphorylierung der EGFR beim Vorliegen der Variante (AA) und damit einhergehend ein längeres Überleben unabhängig von der Therapie.(51) Wir untersuchten in unseren Arbeiten inwieweit der Polymorphismus als prädiktiver Marker für den Einsatz von Cetuximab in Frage kommt. Wir konnten in einer Patientenkohorte unter Behandlung mit Cetuximab und Docetaxel keine Assoziation zum Gesamtüberleben erkennen, jedoch einen signifikanten Zusammenhang zwischen dem Auftreten der Hauttoxizität und einem verminderten Risiko der Tumorprogression. Funktionell wurden unsere Arbeiten später untermauert durch die Untersuchungen, dass Cetuximab eine geringere Bindungsaffinität beim Vorliegen der Variante aufweist

mit der Konsequenz, dass eine verringerte Inhibition der Signalweginhibition mit dem Ausbleiben von Apoptose stattfindet.(52) Die Autoren schlußfolgerten, dass ein optimierter Antikörper wie z.B. Cetugex diese Unterschiede aufheben könnte, was sich in der klinischen Praxis jedoch nicht belegen ließ.(53)

Primäre und sekundäre Resistenzmechanismen

Amphiregulin (AREG) und Epiregulin (EREG) sind Liganden am EGF-Rezeptor. Nachdem in Zelllinienmodellen eine Korrelation zwischen Amphiregulin Expression und der Wirkung von EGFR Inhibitoren Gefetinib und Cetuximab gezeigt werden konnte, entstand die Hypothese, dass eine hohe Amphiregulin Expression prädiktiv für das Ansprechen einer EGFR gerichteten Therapie sein könnte.(54) Diese Hypothese wurde zunächst wiederum bei Patienten mit kolorektalen Karzinomen untersucht. 110 Patienten wurden mit einer Cetuximab Monotherapie behandelt. In Genexpressionsstudien mit einem unsupervised clustering zeigte sich, dass die Patienten mit Tumoren und höchsten AREG und EREG Expressionswerten auch ein deutlich verlängertes progressionsfreies Überleben und eine höhere Rate an Erkrankungskontrolle aufwiesen.(27) Inwieweit diese Daten auf eine Patientenkohorte von Kopf-Hals Karzinomen übertragbar sind, wurde in der CETAX Kohorte untersucht. In unserer Kohorte war eine hohe Proteinexpression mit einem geringeren Ansprechen auf die Kombination von Docetaxel und Cetuximab assoziiert. Die Ergebnisse des Kolonkarzinoms konnte somit nicht reproduziert werden. Inwieweit die Kombinationstherapie von Docetaxel und Cetuximab den Effekt von Amphiregulin beeinflußt oder aber die methodischen Unterschiede Protein- vs Genexpression eine Rolle spielen, bleibt Gegenstand der wissenschaftlichen Diskussion.

Im Gegensatz zu kolorektalen Karzinomen, bei denen Mutationen im Ras-Raf Signalweg, die zur intrinsischen Aktivierung führen, als negativ prädiktiv für eine Wirksamkeit von Cetuximab identifiziert werden konnten, treten diese Mutationen im Kopf-Hals Karzinom nur sehr selten auf. Die Mutationsfrequenz von Kras im Kolonkarzinom liegt bei ca. 40%, die für BRAF Mutationen bei ca 15%, weitere weniger häufig auftretende Mutationen finden sich in NRAS (5%) oder HRAS (3%).(55) Allen gemeinsam ist eine verminderte Wirksamkeit der EGFR Blockade durch eine mutationsbedingte Aktivierung des MAPK Signalwegs.(56) Bei Kopf-Hals Karzinomen liegt die Mutationsfrequenz von HRAS zwischen 5-9%. Damit gilt dieses Gen als am

häufigsten mutiert innerhalb des Ras-Raf-MAPK Singalwegs. Tibifarnib, eine Substanz die spezifisch an mutiertes HRAS bindet und den Signalweg inhibiert, befinden sich gegenwärtig in der Entwicklung.(57) Ein weiterer beschriebener primärer Resistenzmechanismus gegenüber Cetuximab besteht im Nachweis von aktivierenden PI3K Mutationen sowie der Amplifikation des Signalwegs.(58) Die Inhibition der PI3-Kinase zeigte eine relevante Verbesserung des Überlebens. Problematisch waren hohe Toxizitäten und das Fehlen definierter prädiktiver Biomarker, die eine Zulassung von Buparlisib nicht sinnvoll erscheinen ließen.(59) Eine biologisch sinnvolle, gemeinsame Blockade von PI3K und EGFR wurde in klinischen Studien bislang nicht untersucht.

Weitaus häufiger als die dargestellten primären Resistenzmechanismen gegenüber Cetuximab, sind sekundäre Resistenzen, die im Zuge der Antikörpertherapie entstehen. (60)
Die Blockade des EGFR führt zur Aktivierung alternativer Signalwege bzw. Expression alternativer Rezeptoren. Es konnte gezeigt werden, dass die Her-3 Expression und Dimerisierung mit Her-2 nach EGFR Blockade signifikant zunimmt und mit einer Aktivierung von PI3K und Akt einhergeht. Duale Antikörper gegen Her-3 und gleichzeitig EGFR befinden sich gegenwärtig in der klinischen Testung um diesen Resistenzmechanismus zu untersuchen.(61)
Das komplexe Netzwerk des EGFR mit verschiedenen Signalwegen, die interagieren erhöht die Wahrscheinlichkeit, dass ein Tumor alternative Signalwege aktiviert um zu überleben, wenn es zur Blockade eines Rezeptors oder eines Signalwegs kommt. Es ist in diesem Zusammenhang viel wichtiger molekulare Signaturen zu definieren, die einen Tumor charakterisieren und Therapien entsprechend dieser Signaturen auszuwählen.

Molekulare Subtypen von Kopf-Hals Karzinomen

Verschiedene Arbeitsgruppen haben unabhängig voneinander molekulare Subtypen von Kopf-Hals Karzinomen definiert. Eine einheitliche Definition dieser molekularen Subtypen, vergleichbar dem Kolonkarzinom, hat bislang nicht stattgefunden. Allen Gruppen gemeinsam ist aber die Definition eines Basaltyps, der eine hohe EGFR Signalwegaktivierung zeigt. Neben diesen wurde ein Classical Typ mit hoher Aktivierung der Zellzyklusgene definiert und ein Inflamed/Mesenchymaltyp mit einer

entsprechenden Expression mesenchymaler Gene von Vimentin und MMP-9 bei gleichzeitig verminderter Expression epithelialer Gene.(28) Neben der biologischen Beschreibung fand eine prognostische Einordnung verschiedener molekularer Subtypen statt. Interessant sind prädiktive Assoziationen. Seiwert et al. zeigte, dass bei hoher Expression inflammatorischer Marker ein Ansprechen auf Immuntherapie wahrscheinlich ist und eventuell als Prädiktion genutzt werden könnte.(62) In unseren eigenen translationalen Forschungen evaluierten wie das Ansprechen von Cetuximab in Abhängigkeit des molekularen Subtyps. Wir konnten zeigen, dass Tumore, die eine Basaltypsignatur aufwiesen, deutlich besser auf eine EGFR Blockade reagierten, als Tumore, die eine mesenchymale Signatur aufwiesen. Basaltyptumore zeigten im Einklang mit den bisherigen Publikationen ein hohes Maß an Signalwegaktivierung des EGFR.

Durch die Bestimmung von Genexpressionsprofilen zur Klassifikation des molekularen Subtyps eines Tumors und nicht durch die Bestimmung einzelner Biomarker kann zukünftig eine Therapie entsprechend der Tumorbiologie selektiert werden, um dem Behandlungsziel einer gut verträglichen, wirksamen und gleichzeitig kosteneffizienten Therapie näher zu kommen.

Zusammenfassung

Die Blockade des EGF Rezeptors ist fester Bestandteil in der Therapie von Kopf-Hals Plattenepithelkarzinomen. Trotz zahlreicher klinisch, translationaler und präklinischer Forschungen konnte bis zum heutigen Tag kein prädiktiver Biomarker für die Behandlung von Kopf-Hals Karzinomen validiert werden.

Die dargestellten Arbeiten zeigen sehr gut die Chancen und gleichzeitig die Limitationen von translationaler Forschung mit dem Ziel der Definition prädiktiver Biomarker auf. Patientenproben sind in der Regel FFPE fixiert, bergen den Nachteil der z.T. langen Lagerung einhergehend mit Nukleinsäuredegradierung und sind nur für einen Teil interessanter Fragestellungen zu gebrauchen. Weiterhin findet in der Regel eine multimodale Behandlung statt, die den Stellenwert eines einzelnen biologischen Charakteristikums, wie in den Arbeiten beispielhaft an Amphiregulin oder EGFRvIII Expression gezeigt, in der Interpretation erschweren.

Präklinische Modelle, die die Erkrankungssituation 1:1 widerspiegeln, gibt es nicht. Ein präklinisches Modell, dass nah am Ausgangstumor bleibt, ist das PDX Modell. Es

konnte gezeigt werden, dass eine grosse Zahl von Kopf-Hals Tumor Xenograftmodellen sehr gut geeignet sind, Hypothesen zu generieren. Eine Validierung der im PDX Modell gefundenen Assoziation zwischen dem molekularen Subtyp eines Basaltyp und dem Ansprechen auf Cetuximab erfolgt zum gegenwärtigen Zeitpunkt an einer klinischen Kohorte.

Um die Behandlung von Kopf-Hals Tumorpatienten nachhaltig zu verbessern brauchen wir aus meiner Sicht drei Dinge:

1. Präklinische Studien in Modellen, die die Tumorbiologie und molekulare Subtypen repräsentieren
2. Hypothesen getriebene klinische Studien mit der zwingenden Notwendigkeit Gewebe zu sammeln und zu annotieren und
3. innovative Medikamentenentwicklungen mit zielgerichteter Wirkung auf die Tumorzellen unter Schonung des gesunden Gewebes.

Werden diese Dinge in Forschungsnetzwerken verfolgt, die einen Informations- und Materialaustausch regeln, werden wir die Behandlung von Krebserkrankungen relevant verbessern.

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Abkürzungen

5-FU – 5 Fluoruracil

ADCC - antibody-dependent cell-mediated cytotoxicity,

AREG – Amphiregulin

BRAF - englspr. Abk. für ***r****apidly* ***a****ccelerated* ***f****ibrosarcoma*

CA-SSR – CA-Single Sequence Repeats

CDK - Cyclin-abhängige Kinasen

CML – chronisch myeloische Leukämie

CRC – Kolonkarzinom

EGF – epidermaler Wachstumsfaktor

EGFR – epidermaler Wachstumsfaktorrezeptor

EREG – Epiregulin

FOLFOX – 5 Fluoruracil, Leukovorin, Oxaliplatin Medikamentenkombination

Her - human epidermal growth factor receptor

IgG1 - Immunglobulin G1

MAPK – englspr. Abk. für mitogen activated protein

MTOR – englspr. Abk. für mammalin target of rapamycin

NK-Zellen – natural killer Zellen

PDX – patient derived xenograft

PI3K - Phosphoinositid-3-Kinase

Ras - Rat *s*arcoma

RNA - Ribonukleinsäure

TCGA - the Cancer Genome Atlas

TGF-alpha - tumor growth factor alpha

TKI -Tyrosinkinase

Danksagung

Erklärung

Gemäß § 4 Abs. 3 (k) der Habilitationsordnung der Medizinischen Fakultät Charité – Universitätsmedizin Berlin erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur guten wissenschaftlichen Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

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