Spotlight on Clinical Response

Molecular determinants of response to matuzumab in combination with paclitaxel for patients with advanced non-small cell lung cancer

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Abstract

Antibodies targeting epidermal growth factor receptor (EGFR) have proven to be effective in patients with non-small cell lung cancer (NSCLC) that express EGFR. We recently published a phase I study of weekly matuzumab plus paclitaxel. This therapy was well tolerated and showed clinical responses in the majority of patients. Although matuzumab displays potent antitumor activity in some patients, not all patients respond well to treatment. Whether dysregulation of EGFR-mediated pathways precludes or sensitizes cells to paclitaxel is unknown. We sought to determine molecular predictive factors for therapy response in a phase I/II study patient cohort treated with matuzumab ± paclitaxel. Twenty-three cases [including one complete response (CR), three partial responses (PR), 10 stable diseases (SD)] were screened using immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), PCR/sequencing and denaturing wave high performance liquid chromatography (D-HPLC) for expression, amplification, and mutation status of EGFR and downstream signaling pathways. All patients with PR or CR displayed an either high overall or single-cell EGFR expression in the majority of cells. In addition, all of the moderate responders, who achieved SD after at least two cycles of therapy, showed diffuse EGFR expression rates and/or strong single-cell EGFR expression. In contrast, 44% of the nonresponders showed low overall or single-cell EGFR expression levels. No low-expressing EGFR cases were present within the responder group. In addition, among patients with a gain-of-function mutation in KRAS primary therapy failure and/or short responses to therapy were observed. Our data suggest that EGFR expression and KRAS mutation status is predictive for clinical response to matuzumab ± paclitaxel in patients with advanced NSCLC. [Mol Cancer Ther 2009;8(3):481–9]

Introduction

Among malignancies, lung cancer ranks as one of the most common and lethal. Effective therapeutic options for advanced stages remain limited and cure rates are low (1). Approximately 40% to 80% of cases of non-small cell lung cancers (NSCLC) have over-expression of epidermal growth factor receptor (EGFR) protein: EGFR-positive cases are associated with an increased risk of metastases and decreased overall survival (2–4), and the status of EGFR activation (i.e., phosphorylation) predicts for a significantly poorer prognosis in stage I NSCLC patients who undergo surgical resection (5).

Several genomic mechanisms of constitutive activation of EGFR gene have been reported. Historically, the first of these to be described was EGFRvIII, a constitutively active genomic deletion variant of EGFR. Most commonly,

Received 1/2/09; accepted 1/26/09; published OnlineFirst 3/10/09.

Grant support: Grant support in part by a Merit Review Grant from the Department of Veterans Affairs (MCH), the Doris Duke Charitable Foundation (MCH), and the Deutsche Krebshilfe Foundation (MMS). One of the authors (RK) is employed by a company (Merck KGaA) whose product was studied in the present work.

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doi:10.1158/1535-7163.MCT-08-1068

EGFRvIII genomic deletion results in the synthesis of an aberrant mRNA lacking 801 bases that encode amino acids 6–273 (exons 2–7) of the receptor's extracellular domain. The EGFRvIII mutation is frequently found in association with glioblastomas (6), but has also been reported to be found in up to 16% to 39% of NSCLC (predominantly squamous cell carcinomas) (refs. 7, 8).

In addition, several groups have described the presence of intragenic constitutively activating EGFR mutations in the tyrosine kinase (TK) domain that are found in ∼2% to 25% of NSCLC patients (predominantly adenocarcinomas, especially with bronchioalveolar carcinoma histologic features). Interestingly, this type of NSCLC occurs almost exclusively in patients without any smoking history (9–11). The presence of EGFR mutations in the TK domain of EGFR enhances the catalytic activity of the kinase (12) and strongly predicts for clinical responses to small molecule EGFR tyrosine kinase inhibitors (TKI) such as gefitinib (9).

Another approach to targeting EGFR in lung cancer has been the development of monoclonal antibodies with neutralizing activity. Matuzumab (13), cetuximab (14), and ABX-EGF (15) have proven to be effective in clinical studies enrolling patients with a variety of EGFR-expressing tumors. Recent *in vitro* data suggest that cetuximab potently targets TKI-sensitive as well as resistant EGFR TK mutant isoforms (16–18), but substantial differences are observed in clinical response rates (14). To date, the sole positive predictive factor for response to cetuximab-based therapies in NSCLC is an increase in EGFR gene copy number (≥4) detected by fluorescence *in situ* hybridization (FISH) analysis (19). Whether this applies to antibodies with an unrelated EGFR-binding epitope (such as matuzumab) (ref. 20) is not known.

The PI3K/AKT (21), STATs (22), and the RAS/MAPK (23) pathways, are major signaling networks linking EGFR activation to cell proliferation and survival (24). Theoretically, dysregulation of EGFR downstream effectors might predict for resistance or sensitization to anti-EGFR targeted therapy. Gain-of-function mutations of the KRAS GTPase occur in 10% to 30% of NSCLC and are strongly associated with adenocarcinomas arising in former or current smokers (10, 25). In several studies of NSCLC, the presence of a KRAS mutation correlated with poor prognosis (26, 27). In addition, autoactivating mutations of PI3K-isoform PIK3CA, have been reported as oncogenic in a broad range of malignancies, including NSCLCs (28, 29). The notion that PIK3CA mutations might predict for therapy failure of EGFR-targeted antibodies observation has been further supported using an *in vitro* lung cancer model (30).

In the current study, we analyzed tumor samples from a NSCLC patient cohort for molecular predictors of response to EGFR-targeted therapy. The patients were treated within a phase I/II trial (13) with paclitaxel and matuzumab (formerly EMD72.000), a fully humanized antibody potently targeting EGFR (31). Paclitaxel, a mitotic inhibitor, interferes with spindle formation leading to G2/M phase cell cycle arrest and induction of apoptosis (32). Of note, there is evidence that paclitaxel induces phosphorylation of EGFR and activation of EGFR-mediated pathways via AKT and ERK/MAPK in cancer cells (33–35). This is believed to function as a potential cellular survival mechanism after chemotherapy exposure. Theoretically, EGFR inhibitors might enhance antitumor activity after ''EGFR-sensitization'' with paclitaxel. *In vitro* studies have shown an augmented antitumor effect of paclitaxel when it is combined with an EGFR inhibitor (35). Conversely, dysregulated activation of EGFR-mediated pathways, as often occurs in NSCLC, might prevent response to paclitaxel and/or an EGFR inhibitor like matuzumab. In our study, we sought to determine molecular predictive factors for therapy response to combination therapy with matuzumab and paclitaxel.

Materials and Methods

Study Design

Twenty-three archival samples of paraffin-embedded tissue from consenting patients treated in the EMD72.000 phase I/II trial of weekly matuzumab (dose-escalating 100 to 1,200 mg) and paclitaxel (175 mg/m^2 q21days) were analyzed (ethics committee vote 117/2,001). Matuzumab (formerly EMD72.000), from Merck KGaA (Darmstadt, Germany), is a humanized monoclonal antibody (31) that binds to the extracellular domain III of EGFR in a region that is nonoverlapping from the cetuximab binding site and inhibits ligand-mediated *in vitro* kinase activation (20). The clinical study was conducted as a monocenter trial at the University Hospital of Tübingen/Germany from 2001 to 2005 and was sponsored by Merck KGaA. The clinical data of the phase I study were published recently by our group [Kollmannsberger and colleagues (13)]. Table 1 shows the patient characteristics and response rates of all assessable patients. Biomarker studies were performed in a blinded fashion without knowledge of clinical outcome. Tumors for sequencing were selected on the basis of availability of suitable specimens rather than on response or other clinical characteristics. Patients with missing values for a variable were excluded from any analysis involving that variable.

Immunohistochemistry

A reference pathologist (SS) assessed EGFR expression by immunohistochemistry (IHC) in each case prior to study entry. IHC staining of the tumor tissue array samples was performed by using the EGFR pharmDx kit (DakoCytomation, Carpinteria, CA) as previously described (36). Only patients with detectable EGFR expression (minimum of 5% of all tumor cells) were included in the clinical study. Expression status was classified as 1+ (faint or barely perceptible partial staining of the membrane), 2+ (weak to moderate complete membrane staining or strong partial staining of the membrane), or 3+ (strong complete membrane staining in at least 10% of tumor cells).

FISH Analysis

BAC clone CTD-2026n22 containing the genomic DNA sequence of the EGFR gene (Human 32K BAC Re-Array,

CHORI),[8] was used as a template for gene-specific PCR. Eight fragments, totaling approximately 10 kb, spannin-g exon 2–7, were PCR amplified. PCR products were pooled and labeled with biotin-16-dUTP (Roche Diagnostic, Basel, Switzerland) by nick translation (Invitrogen, Carlsbad, CA), according to the supplier's instructions. The second probe SPoT-Light EGFr, detecting the whole locus, was purchased from Zymed (San Francisco, CA). Frozen tissue sections (4 μM) were pretreated according to the manufacturer's protocol (Frozen TissuePrep for FISH, Vysis, Downers Grove, IL). FISH analysis was performed as previously described (37). More than 50 interphase nuclei are examined for each sample.

PCR

All samples were reviewed by a reference pathologist (CLC). Tumor tissue with the highest purity was selected from paraffin blocks using a punch biopsy, and genomic DNA was extracted using a DNAeasy Tissue kit (Qiagen, Valencia, CA). EGFR exons 18, 19, 20, and 21 were amplified using the following primer pairs: 5′-GCTGAGGTGACCCTTGTCTC-3′ (EGFR sense primer, exon 18), 5′-ACAGCTTGCAAGGACTCTGG-3′ (EGFR antisense primer, exon 18), 5′-AGCATGTGGCACCATCTCAC-3′ (EGFR sense primer, exon 19), 5′-AGACATGAGAAAAGGTGGGC-3′ (EGFR antisense primer, exon 19), 5′-CCTCCTTCTGGCCACCAT-3′ (EGFR sense primer, exon 20), 5′-CCTGATTACCTTTGCGATCTG-3′ (EGFR antisense primer, exon 20), 5′-AGCAGGGTCTTCTCTGTTTCAG-3′ (EGFR sense primer, exon 21), 5′-CCTGGTGTCAGGAAAATGCT-3′ (EGFR antisense primer, exon 21). KRAS exon 1 and 2 was amplified as follows: 5′-TTAACCTTATGTGTGACATGTTCTAA-3′ (KRAS sense primer, exon 1), 5′- TCATGAAAATGGTCAGAGAAACC-3′ (KRAS antisense primer, exon 1), 5′-TTTTTGAAGTAAAAGGTGCACTG-3′ (KRAS sense primer, exon 2), 5′-TGCATGGCATTAGCAAAGAC-3″ (KRAS antisense primer, exon 2). PI3K exons 9 and 20 were amplified as follows: 5′-TTGAAAATGTATTTGCTTTTTCTGT-3′ (PI3K sense primer, exon 9), 5′-CATGTAAATTCTGCTTTATTTATTCCA-3′ (PI3K antisense primer, exon 9), 5′-CATTTGCTCCAAACTGACCA-3′ (PI3K sense primer, exon 20), 5′-GGTCTTTGCCTGCTGAGAGT-3′ (PI3K antisense primer, exon 20). PCR amplification of genomic DNA was performed using 500 ng of DNA (38).

Denaturing Wave High Performance Liquid Chromatography

Five to 20 μL aliquots of each PCR reaction were assessed for EGFR, KRAS, or PIK3CA mutations using a Transgenomic WAVE high performance liquid chromatography (HPLC) system (Trangenomic Inc, Omaha, NE). Samples were run at 50°C (EGFR, exons 19 and 20) to check for length mutations or at 62°C for EGFR exon 18, 59.4°C for EGFR exon 19, 63°C for EGFR exon 21, 58.1°C for KRAS exon 1, 58.5°C for KRAS exon 2, 55.4°C for PIK3CA exon 9, and 57.5°C for PIK3CA exon 20 for the screening for point mutations. In addition, amplimers were bi-directionally sequenced on an ABI 310 sequencer using the BigDye terminator kit (38).

Results

Patient Cohort

We performed a retrospective, exploratory analysis using archival patient samples to identify biomarkers predictive of clinical response in a cohort of patients treated in a phase I/II study testing the humanized monoclonal EGFR antibody matuzumab in combination with the mitotic inhibitor paclitaxel. The results from the phase I portion of this study, including patients treated with the 200-, 400-, and 800-mg doses of matuzumab, were previously published (13). In our patient cohort, including 23 eligible patients from the phase I/II, clinical activity was seen in 61% (14/23) of patients, including one complete response (CR, 1/23, 4%), three partial responses (PR, 3/23, 13%), and 10 cases with stable diseases (SD, 10/23, 44%) as defined by Response Evaluation Criteria In Solid Tumors (RECIST) criteria for at least two cycles of therapy [as assessed by computed tomography (CT) imaging]. Nine of the 23 patients (39%) had progressive disease at the end of two cycles of therapy (Table 1).

EGFR Expression Profiling

As a prerequisite of study entry, only patients whose tumor had EGFR-positive IHC testing were included in the clinical trial. All patients who achieved CR/PR had either diffuse expression of EGFR (defined as >70% of cells staining positively) (3/4, 75%), and/or strong single-cell EGFR expression (minimum ''2+'' as determined by IHC) in the majority of cells (3/4, 75%). In addition, all of the patients with stable disease for at least two cycles of therapy (10/10, 100%) had tumors with diffuse EGFR expression (8/10, 80%) and/or strong single-cell EGFR expression profiling (8/10, 80%). In contrast, 44% (4/9) of the nonresponders showed low levels of overall or single-cell EGFR expression. Tumors with ''low-level EGFR'' expression were not found within the group of responding patients (Table 1).

EGFR Genomic Amplification Status

Theoretically, EGFR amplification might result in abnormal receptor activation and contribute to cellular proliferation. In addition, in a preclinical model of lung cancer, EGFR mutational status was positively associated with EGFR genomic amplification (39). We performed FISH analysis for EGFR amplification on 14 evaluable patient samples. Two of the patients [14% (2/14), Fig. 2], had >five copies of EGFR (Table 1; Fig. 1). The first patient was an elderly (70 years old) woman with metastatic adenocarcinoma (T4N3M1). FISH analysis revealed five to six copies of EGFR per tumor cell (Fig. 3). Interestingly, using IHC, only ~5% of tumor cells displayed any significant EGFR expression, but with a 2+ single cell expression rate. The patient had a minor response to combination therapy with M (400 mg) + P: RECIST-defined SD for seven cycles. The tumor from the second patient, a 48-year-old woman with metastatic adenocarcinoma (T2N0M1), had >10 copies

[8] http://bacpac.chori.org/

Table 1. Patient characteristics and molecular analysis of 23 patients treated with matuzumab weekly, dose-escalating 100 to 1,200 mg, and paclitaxel, 175 mg/m^2, q21days

Patient	Age/ Gender	TNM/AJCC	Smoking history	Histology	Chemonaive	Dose level (mg abs.)/ Cycles (Overall)	Without paclitaxel since cycle	Best response/ after × cycles	Response/ after × cycles
1	61/F	T4 N3 M1/IV	Y	Ad	PRE	200/4	–	SD/2	PD/4
2	61/M	T1 N1 M1/IV	Y	Ad	PRE	200/2	–	PD/2	PD/2
3	70/M	T3 N2 M1/IV	N	Sq	PRE	200/5	–	SD/2	PD/4
4	71/M	T3 N3 M1/IV	Y	Ad	Y	400/4	–	SD/2	PD/4
5	70/F	T4 N3 M1/IV	Y	Ad	Y	400/2	–	PD/2	PD/2
6	62/M	T2 N2 M1/IV	Y	Ad	Y	800/26	–	PR/6	PD/26
7	61/M	Tx Nx M1/IV	Y	Ad	Y	800/12	–	PR/2	PD/12
8	65/M	Tx Nx M1/IV	Y	Sq	Y	800/25	7	CR/2	PD/25
9	65/M	T2 N3 M1/IV	Y	Ad	PRE	800/4	–	PR/2	PD/4
10	63/M	T3 N3 M0/III B	Y	Ad	Y	800/6	1	SD/2	SD/2
11	38/M	T4 N3 M1/IV	Y	Ad	PRE	800/2	1	PD/2	PD/2
12	72/M	T2 N0 M1/IV	Y	Sq	Y	800/3	–	SD/2	N/A
13	76/M	T4 N2 M1/IV	Y	Sq	Y	800/3	–	SD/2	SAE
14	71/F	T2 N2 M1/IV	Y	Ad	PRE	800/2	–	PD/2	PD/2
15	56/M	T2 N0 M1/IV	Y	Sq	Y	1,200/6	1	SD/2	PD/6
16	57/M	T4 N2 M1/IV	Y	Ad	Y	1,200/6	–	SD/2	PD/4
17	48/F	T2 N0 M1/IV	N	Ad	Y	1,200/8	–	SD/2	SD/7
18	48/F	T1 N3 M1/IV	Y	Ad	PRE	1,200/2	–	PD/2	PD/2
19	41/M	N/A/N/A	Y	Ad	PRE	1,200/6	–	PD/2	PD/6
20	78/M	T2 Nx M1/IV	Y	Ad	PRE	1,200/3	–	SD/2	SD/2
21	50/M	T4 N3 Mx/III B	Y	Ad	Y	1,200/2	–	PD/2	PD/2
22	75/F	T4 N2 M0/III B	Y	Ad	Y	1,200/4	–	PD/2	PD/2
23	39/M	T3 N2 M1/IV	N	Ad	PRE	1,200/1	–	PD/2	PD/2

NOTE: Patient data were anonymized. Caucasian population. Median age was 61 years (*SD* ± 12 years). Seventeen male versus six female patients. Most patients had a smoking history. All patients had advanced disease at study entry. Adenocarcinoma was the predominant histological finding. Responders were defined as CR, PR, or SD after a minimum of two cycles. The combination of matuzumab with paclitaxel was safe and showed efficacy in 14/23 (61%) patients (one CR, three PR, 10 SD); some patients had a prolonged response. PD: progressive disease as defined by RECIST criteria with at least 20% increase in the sum of the longest diameter of target lesions.

Abbreviations: F, female; M, male; Y, Yes; N, No; PRE, pretreatment; Ad, adenocarcinoma; Sq, squamous cell carcinoma; CR, complete remission; SAE, severe adverse event; N/A, not applicable; WT, wild-type isoform.

EGFR expression: 1+, faint or barely perceptible partial staining of the membrane; 2+, weak to moderate complete membrane staining or strong partial staining of the membrane; 3+, strong complete membrane staining in at least 10% of tumor cells.

*EFGR-TK Mutations, mutations located in the intracellular tyrosine kinase domain.

†EGFRvIII-Isoform, isoform of EGFR harboring a deletion-mutation in the extracellular domain III.

‡No tissue/no amplification.

§>10 Copies.

‖Deletion exon 19: ELREATS 746–752(V).

¶Five to six copies.

**Point mutation exon 1: G12C/D.

of EGFR per tumor cell. IHC revealed a high overall expression rate of 70% with a moderate to high single cell expression. However, this patient's tumor did not respond to M (1,200 mg) + P (PD).

EGFR Mutation Status

Autoactivating somatic mutations of the TK domain of EGFR serve as an oncogenic mechanism in a subgroup of cases of NSCLC. Notably, EGFR TKIs, such as gefitinib, produced high rates of objective and durable responses in these patients (9). In contrast, there are no valid molecular markers predicting clinical response to monoclonal EGFR antibodies. We screened twenty assessable patient samples for the occurrence of autoactivating EGFR mutations in the TK domain. We found a gain-of-function EGFR mutation in one of the 20 patient samples: a deletion of codons 746 to 752 (ELREATS) in exon 19 of EGFR (Fig. 2). This particular tumor was obtained from a 65-year-old male with metastatic adenocarcinoma treated in the 800-mg arm of matuzumab plus paclitaxel as a second line regimen. The patient had an initial PR, but was documented to have PD after 12 weeks of therapy (Table 1). Of note, this tumor displayed a high overall expression rate but only a rather moderate EGFR expression on per cell basis. Except for the histologic subtype, none of the patient associated factors (female, Asian, nonsmoker) predicting for the presence of an EGFR-activating mutation was present (9).

We also evaluated whether mutations in the extracellular domains of EGFR may predict for response to anti-EGFR antibody therapy. The EGFRvIII deletion variant results in a loss of a portion of the extracellular domain III of EGFR. The kinase activity of the EGFRvIII variant is relatively insensitive to gefitinib or erlotinib (8). Theoretically, conformation

Table 1. Patient characteristics and molecular analysis of 23 patients treated with matuzumab weekly, dose-escalating 100 to 1,200 mg, and paclitaxel, 175 mg/m^2, q21days (Cont'd)

Patient	Responder (CR/PR/SD)	EGFR Expression (%)				EGFR amplification	EFGR-TK mutations*	EGFRvIII isoform †	K-RAS mutations	PIK3CA mutations
		Positive Cells	1+	2+	3+					
1	Y	70%	90%	10%	0%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
2	N	60%	80%	15%	5%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
3	Y	70%	70%	20%	10%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
4	Y	95%	10%	90%	0%	N/A ‡	WT exon 18–21	N/A ‡	N/A ‡	N/A ‡
5	N	70%	0%	40%	60%	Y§	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
6	Y	95%	0%	10%	90%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
7	Y	25%	40%	60%	0%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
8	Y	98%	10%	30%	60%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
9	Y	80%	60%	20%	20%	N	DEL‖ Exon 19	N	WT exon 1, 2	WT exon 9, 20
10	Y	100%	0%	100%	0%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9
11	N	50%	65%	20%	15%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
12	N/A	100%	0%	0%	100%	N/A ‡	WT exon 18–21	N/A ‡	WT exon 1, 2	WT exon 9, 20
13	N/A	60%	30%	70%	0%	N/A ‡	WT exon 18–21	N/A ‡	WT exon 1, 2	WT exon 9
14	N	90%	0%	20%	80%	N/A ‡	WT exon 18–21	N/A ‡	WT exon 1, 2	WT exon 9, 20
15	Y	90%	40%	40%	60%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
16	Y	70%	0%	60%	40%	N/A ‡	WT exon 18–21	N/A ‡	WT exon 1, 2	WT exon 9, 20
17	Y	5%	0%	100%	0%	Y¶	WT exon 18–21	N	PM** G12D	WT exon 9, 20
18	N	80%	40%	60%	0%	N/A ‡	WT exon 18–21	N/A ‡	WT exon 1, 2	WT exon 9, 20
19	N	5%	100%	0%	0%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
20	Y	90%	50%	50%	0%	N/A ‡	N/A ‡	N/A ‡	WT exon 2	N/A ‡
21	N	98%	30%	40%	30%	N/A ‡	WT exon 18–21	N/A ‡	PM** G12C	WT exon 9, 20
22	N	100%	2%	8%	90%	N	WT exon 18–21	N	WT exon 1, 2	WT exon 9, 20
23	N	60%	80%	20%	0%	N/A ‡	WT exon 18–21	N/A ‡	PM** G12D	N/A ‡

changes of the extracellular domains could alter antibody binding as well. Notably, the matuzumab epitope, although located in the domain III of EGFR, is still present in the EGFRvIII variant (20). The EGFRvIII has been found in association with a minority of cases of glioblastoma multiforme (6, 40) or NSCLC (7, 41) In the current study, 14 patients were assessable by FISH analysis for genomic deletions involving exons 2–7 of EGFR. We found no evidence of EGFRvIII deletions in these samples (Table 1, Fig. 1).

KRAS and PIK3CA Mutational Screening

Activating mutations of KRAS are found in a significant minority of NSCLC and are usually mutually exclusive with EGFR mutations (10). Previous studies have identified KRAS-mutant tumors as being almost uniformly resistant to EGFR kinase inhibitors. We performed KRAS genotyping to determine if the presence of KRAS mutation influenced response to the combination of matuzumab ± paclitaxel.

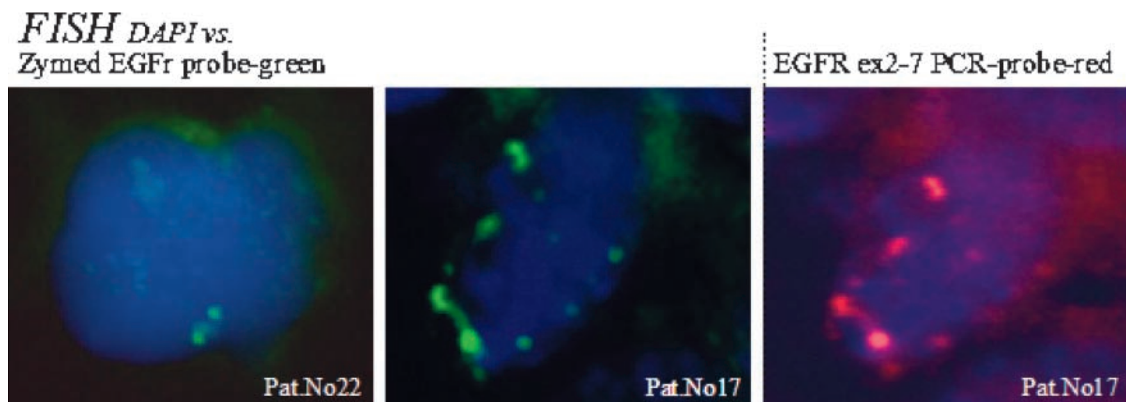

Figure 1. FISH analysis for EGFR amplification (Zymed EGFr probe-green) and truncation of the extracellular III-domain (ex.2 – 7 PCR-probe-red). Tissue from 14 patients was suitable for analysis. Representative FISH results are shown here. Patient 22 has a normal EGFR gene copy number. In contrast, patient 17 has amplification of EGFR with an estimated five to six gene copies. However, using a probe specific for EGFR exons 2 – 7, there is no evidence of any genomic deletion involving the EGFR extracellular domain.

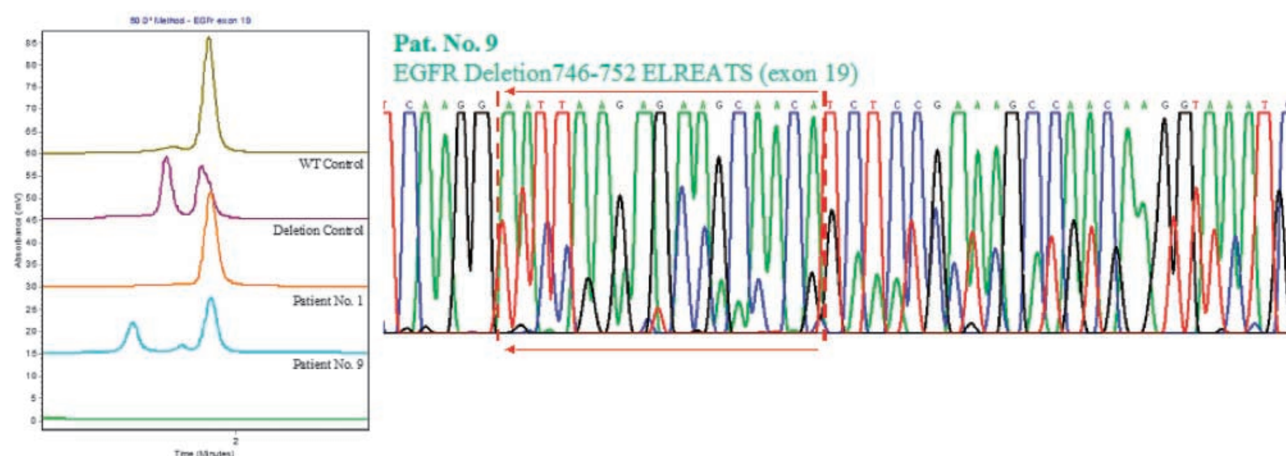

Figure 2. EGFR mutational screening of the TK domain. DNA from tumor tissue from 22 patients was suitable for analysis. DNA was extracted, PCR-amplified, and screened for autoactivating mutations in the EGFR kinase domain (exons 18 – 21). D-HPLC-Wave results are shown comparing one patient sample that was tested wild-type for EGFR (patient 1) and one sample for a patient who harbored an exon 19 mutation (patient 9). This mutation was sequence-confirmed revealing a deletion with a truncation of amino acids ELREATS at codon 746 to 752.

Overall, three of 20 assessable patients (15%) had tumors with KRAS mutations. The mutations occurred at codon 12 (G12D/C), and all were previously described activating mutations (Fig. 3). All three patients had metastatic adenocarcinoma and were treated in the 1,200-mg arm of matuzumab ± paclitaxel as first or second line regimens. Two of the patients were nonsmokers. Two of the patients (both male, 50 years old and 39 years old) were unresponsive to treatment with matuzumab ± paclitaxel (both with documented PD after two cycles of therapy). The third patient had SD as a best response to treatment with matuzumab plus paclitaxel. This patient responded to treatment for 24 weeks of therapy before tumor progression was noted. Notably, this was the same patient who was found to have an EGFR amplification with five to six copies as described above.

PIK3CA mutations have been reported in a subset of patients with NSCLC (28). PIK3CA is a mediator of signaling between EGFR and the AKT/mammalian target of rapamycin (mTor) pathway. Theoretically, PIK3CA mutations may act as an escape mechanism to EGFR targeted therapies, as recently demonstrated in a colorectal cancer cell model (30). Twenty assessable patients were screened for PIK3CA mutations involving exon 9 or 20, which are known hot spots for somatic mutations; none were detected in our patient cohort.

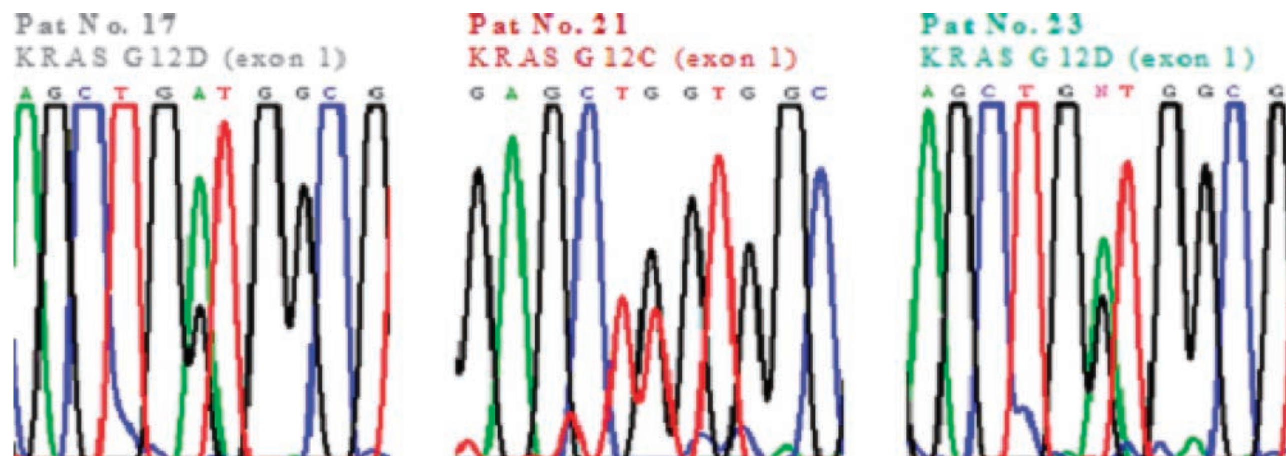

Figure 3. KRAS mutational screening. DNA from tumor tissue was extracted, PCR-amplified, and screened for autoactivating mutations in the KRAS GTPase exon 1 and 2. D-HPLC-Wave results from 20 assessable patients revealed three patients with an exon 1 mutation (data are not shown). These mutations were sequence-confirmed as point mutations of codon 12. In one case the substitution of thymine for guanine led to an exchange of the amino acid Cysteine for Glycine (G12C); two other cases harbored a substitution of adenine for guanine resulting in an exchange of Aspartic acid for Glycine (G12D).

Discussion

Molecular targeted therapy is beginning to be integrated into the routine management of advanced NSCLC. Antibodies, like matuzumab, targeting EGFR have proven to be effective in a subset of patients in single agent as well as combination therapy regimens (13). We recently published the clinical results of a phase I study with matuzumab weekly plus paclitaxel: Administration of matuzumab plus paclitaxel was well tolerated and, in addition, clinical responses were seen in the majority of treated patients. Notably, some patients had durable responses up to 12 months after discontinuation of paclitaxel because of side effects (mostly peripheral neuropathy); these patients received weekly matuzumab monotherapy. Although matuzumab had potent antitumor activity in some patients, response rates varied widely and there are no identified predictive markers.

Mutations in the target receptor, i.e., EGFR, as well as in downstream effectors moderating EGFR signaling are reported to fuel oncogenesis of various NSCLC subtypes. Besides EGFR TK domain- and EGFRvIII-mutations, autoactivating mutations in the GTPase KRAS and in the PI3Kinase isoform PIK3CA are found frequently in NSCLC (25, 26, 28). Theoretically, these activating mutations in signaling pathways downstream of EGFR could diminish tumor responsiveness to EGFR-targeted inhibitory agents. Indeed, existing clinical data suggest that mutations in KRAS or over-expression of the phosphorylated (activated) isoform of AKT predict for a poor response to EGFR inhibition with gefitinib (42).

In our study, we sought to determine whether the clinical response of patients treated with the EGFR antibody matuzumab alone or in combination with the EGFR-pathway sensitizer paclitaxel could be predicted by molecular markers that were previously reported to be involved in the dysregulation of EGFR or EGFR-mediated pathways in NSCLC.

Paclitaxel is an established chemotherapy agent for the treatment of advanced NSCLC and has shown to have synergistic apoptosis-inducing *in vitro* effects when combined with anti-EGFR antibodies (43). Of note, paclitaxel sensitizes EGFR-mediated pathways owing to a transient phosphorylation of EGFR, which is thought to be a potential resistance mechanism of cancer cells to chemotherapy exposure (33, 34). Whether dysregulation of the EGFR-mediated pathways precludes or sensitizes cells to paclitaxel therapy is unknown.

Taken together, our analysis did not reveal a positive predictor of therapy response to matuzumab ± paclitaxel. Notably, patients with a prolonged response rate to matuzumab plus paclitaxel (including those who discontinued paclitaxel because of side effects) expressed wild-type isoforms of EGFR, KRAS, and PIK3CA. In contrast, low level EGFR expression as assessed by immunohistochemistry clearly precludes clinical response to matuzumab, even when administered in combination with the EGFR-sensitizer paclitaxel. In addition, there was a trend for patients with mutation of KRAS or the TK domain of EGFR to have shorter response durations (including primary progressive disease). Our suggestion of a negative predictive value of KRAS mutations for treatment response to matuzumab in NSCLC is supported by observations in colorectal cancer, where gain-of-function mutations of KRAS has been shown in several studies to be highly predictive for lack of response to the anti-epidermal growth factor receptor antibodies cetuximab or panitumumab (44, 45). Recent data further support the hypothesis that KRAS mutations negatively impact the efficacy of receptor TK targeted therapies (46, 47).

In NSCLC xenograft models of tumors with constitutively active EGFR mutations, cetuximab successfully hinders kinase activity with a resultant inhibition of cellular growth (17). This is in contrast to the findings in our clinical study. The reason for these discrepant results is unknown, but could be related to different EGFR-binding sites of cetuximab and matuzumab. These differences also may explain differences in the mechanism of action of these two EGFR-targeted antibodies (20). Larger clinical studies will be needed to clarify the relationship of EGFR TK mutations and response to matuzumab in NSCLC patients.

Interestingly, despite the fact that our biomarker analysis focused on factors that might relate to response to matuzumab treatment, we found evidence that low EGFR expression and KRAS mutations predict for inferior clinical response to therapy with matuzumab alone, as well as in combination with paclitaxel. In contrast, there are no data to suggest that these markers would be predictive for response to single agent paclitaxel treatment. This observation may be due to the EGFR-sensitizing potency of paclitaxel: paclitaxel-triggered transient phosphorylation of EGFR (34) mimics ligand-induced activation of EGFR, which is potently blocked by matuzumab (20). Consistent with our theory, it was recently suggested in a patient cohort with advanced colorectal cancer treated with cetuximab, that disease control was more likely to be reached for tumors with high expression of the EGFR ligands amphiregulin and epiregulin (48). These data support the hypothesis that activation of EGFR sensitizes cancer cells to anti-EGFR therapy.

In conclusion, EGFR-targeted therapy with matuzumab alone or in combination with paclitaxel has promising clinical activity. Historically, response rates of 10% to 15% are seen when using single agent paclitaxel to treat advanced NSCLC (49, 50). In contrast, the clinical benefit rate for matuzumab ± paclitaxel in our study cohort was significantly higher (~60%), suggesting an additive or synergistic effect of this drug combination. KRAS mutations screening and low EGFR expression may serve as negative predictive factors for therapy response to matuzumab ± paclitaxel. Further exploration of the role of this novel combination for treatment of patients with advanced NSCLC and detectable intermediate or high EGFR expression level is part of an extended phase II study.

Disclosure of Potential Conflicts of Interest

R. Kurek, employee of Merck KGaA. No other potential conflicts of interest were disclosed.

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