[CANCER RESEARCH 64, 9139–9143, December 15, 2004]

An Epidermal Growth Factor Receptor Intron 1 Polymorphism Mediates Response to Epidermal Growth Factor Receptor Inhibitors

Maria L. Amador,[1] Darin Oppenheimer,[1] Sofia Perea,[1] Anirban Maitra,[1] George Cusati,[1] Christi Iacobuzio-Donahue,[1] Sharyn D. Baker,[1] Raheela Ashfaq,[2] Chris Takimoto,[3] Arlene Forastiere,[1] and Manuel Hidalgo[1]

[1]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, Baltimore, Maryland; [2]University of Texas Southwestern Medical Center, Dallas, Texas; and [3]Cancer Therapy and Research Center, San Antonio, Texas

ABSTRACT

This study tested the hypothesis that the number of CA single sequence repeat (CA-SSR) in the intron 1 of the epidermal growth factor receptor (*egfr*) gene, which affects transcription efficiency of the gene, is associated with the response to *EGFR* inhibitors. To this end, we determined the number of CA dinucleotides in the intron 1 of the *egfr* gene in a panel of 12 head and neck cancer cell lines that lack *egfr* gene amplification and measured the expression of *EGFR* (mRNA and protein), as well as response to *EGFR* inhibition. Cells with lower number of CA dinucleotides in the CA-SSR had higher expression of the EGFR gene and protein and were more sensitive to the inhibitory effects of erlotinib, a small molecule inhibitor of the EGFR tyrosine-kinase. Phenotypic modification by silencing *EGFR* mRNA expression in a susceptible cell line induced resistance to the drug. The number of CA dinucleotide was equivalent in genomic and tumor DNA obtained from 30 patients with head and neck cancer. In a clinical study in colorectal cancer, subjects with lower number of CA dinucleotide frequently developed skin toxicity, a feature that is related to the antitumor activity of this class of drugs. These results suggest that polymorphic variations in the intron 1 of the *egfr* gene is associated with response to *EGFR* inhibitors and may provide an explanation as to why the development of skin toxicity is associated with a favorable outcome in patients treated with these agents.

INTRODUCTION

The epidermal growth factor receptor (EGFR) is a M_r 170,000 transmembrane glycoprotein encoded by a gene located on the short arm of the human chromosome 7 (1). The EGFR plays a critical role in the control of processes occurring during oncogenesis and tumor progression, such as cell survival, proliferation, and metastasis. The receptor is overexpressed in an extensive range of solid tumors, and this alteration has been associated with advanced stage of disease, poor prognosis, and development of resistance to chemotherapy, radiotherapy, and hormone therapy (2–5). Because of its prevalence in malignant diseases and its crucial role in tumorigenesis, targeting the EGFR is a rational approach for cancer treatment. Several compounds, including monoclonal antibodies directed against the extracellular domain of EGFR, and small molecule inhibitors of the tyrosine-kinase (TK) activity of the receptor have entered clinical trials. Single-agent phase II studies of these compounds have shown response rates in the range of 5 to 15% in selected tumors types (6–12). Individual variability in response to *EGFR* inhibitors has been observed. A significant interest exists in defining factors that could be used to identify patients more likely to respond to EGFR inhibitors so that treatment can be targeted to the appropriate subjects. Recent studies in patients with non–small-cell lung cancer (NSCLC) have found that patients with somatic mutations in the kinase domain of the receptor are extremely susceptible to these drugs and achieved substantial and prolonged responses when treated with gefitinib, a small molecule inhibitor of the EGFR TK (13, 14). These mutations are more frequent in nonsmoker female patients of Japanese origin with adenocarcinomas, explaining the previously observed clinical features of responding patients. The mutations, however, have only been found in ≈10% of patients with NSCLC, a figure well below the ~30 to 40% of patients who appear to benefit from these drugs in large clinical trials (6, 7). In addition, no mutations have been found in tumor other than NSCLC, which are also susceptible to the drug such as squamous cell carcinoma of the head and neck (8, 15).

An important observation in the clinical studies conducted with these drugs have been the lack of association between expression of the EGFR, as determined by immunohistochemistry, and clinical benefit (6, 7, 15). However, trials conducted with both monoclonal antibodies and small molecules inhibitors of the EGFR TK have shown a striking correlation between the development of cutaneous rash, the prototypical toxicity of the drugs, and response to the drugs as manifested by objective responses and overall survival in several tumors types (8, 11, 16). Why this occurs is not known. One explanation could be pharmacological: subjects with higher plasma levels of the drug attain greater toxicity and antitumor response. Available studies, however, do not support this notion because there is no association between plasma levels of the drug and the development of rash (15). Another possibility is genetic differences among individuals. In this study, we have tested the hypothesis that genetic variations in the *egfr* gene shared between normal and malignant tumors could explain the observed clinical association. Several studies have revealed that polymorphic variations in genes encoding drug targets affect the response and toxicity to therapeutic agents (17–22). The *egff* gene contains a highly polymorphic sequence in intron 1, which consists of a variable number of CA dinucleotide repeats ranging from 9 to 21 (23). This sequence has been shown to affect the efficiency of gene transcription such that subjects or cell lines with a greater number of CA repeats have lower levels of mRNA and protein expression (24, 25). This combined set of indirect observations led us to formulate the hypothesis that the number of CA dinucleotide repeats in the *egfr* gene influences the response to anti-EGFR–targeted therapies.

MATERIALS AND METHODS

Cell Lines. Thirteen cell lines were used in this study. HN06, HN011, HN012, HN013, HN019, HN022, HN028, HN029, and A431 cell lines were kindly provided by Dr. David Sidransky's laboratory at Johns Hopkins University (Baltimore, MD). CCL-30, Detroit, Hep2, and CAL27 were obtained from the American Tissue Culture Collection (Manassas, VA). The EGFR-rich, EGFR TK inhibitors susceptible A431 cell line in which the number of CA dinucleotide repeats within the intron 1 has been previously reported were

Received 3/23/04; revised 9/1/04; accepted 10/21/04.

Grant support: NIH Grants P50 CA58184-09, R21CA99209, and P30 CA06973. M. Amador is supported by grant 01/9536 from the Instituto de Salud Carlos III, Madrid, Spain. A. Maitra is supported by a Johns Hopkins Clinician-Scientist Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: D. Oppenheimer and M.L. Amador contributed equally to this work.

Requests for reprints: Manuel Hidalgo, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Baunting and Blaustein Cancer Research Building, 1650 Orleans Street, Room 1M88, Baltimore, MD 21231. Phone: (410) 502-9746; Fax: (410) 614-9006; E-mail: mhidalg1@jhmi.edu.

used as an internal control (26). Cells were grown as monolayer culture in different media (Eagle's minimum essential medium for CCL-30, Hep2, and Detroit; DMEM for A431 and CAL27; and RPMI 1640 for the rest of the cell lines). Media were supplemented with 10% of fetal bovine serum and 2.5 mg of penicillin/streptomycin. Cells were maintained at 37°C in a fully humidified atmosphere of 5%CO_2 in air.

Drugs. Erlotinib (OSI-774, Tarceva) was provided by OSI Pharmaceuticals (Melville, NY). Stock solutions were prepared in DMSO and stored at −20°C.

Growth Inhibition Studies. *In vitro* drug sensitivity to concentrations of erlotinib ranging from 0 to 30 μmol/L was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, St. Louis, MO) dye conversion at 570 nm following the manufacturer's instructions. Briefly, cells were trypsinized, seeded at 5×10^3 cells/well in a 96-well plate, and allowed to grow for 24 hours before treatment, increasing concentrations of erlotinib in the presence of 10% fetal bovine serum. All experiments were done in triplicate and were independently repeated at least three times. After a 96-hour period of treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (5 mg/mL in PBS) solution were added to each well. After 3 hours of incubation at 37°C, cells were lysed by the addition of DMSO.

ELISA Assay. An ELISA was used for quantification of EGFR (Oncogene Research Products, San Diego, CA) as per the manufacturer's instructions.

DNA Extraction and Fluorescent Microsatellite PCR Analysis. DNA was extracted from cell pellets with the Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA). Thereafter, 40 ng of genomic DNA were PCR amplified with 5′-FAM–labeled forward EGFR primer (5′-GGGCTCACAGCAAACTTCTC-3′) and unlabeled reverse EGFR primer (5′-AAGCCAGACTCGCTCATGTT-3′). PCR conditions are available on request. Genotypes were resolved on the Applied Biosystems ABI377 genetic analyzer for allele length determination. In addition, PCR product from A431 was directly sequenced with the forward primer to confirm the number of CA dinucleotide repeats (25).

Quantitative Real-time Reverse Transcription-PCR Analysis. Total RNA was extracted from cell lines with the RNeasy Mini kit (Qiagen) and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA. Relative quantification of *egfr* mRNA was done with an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) with Sybr green as the fluorophore and previously described exonic primers (27). Glyceraldehyde-3-phosphate dehydrogenase mRNA was measured as control gene and relative expression calculated with the $2^{-\Delta Ct}$ method, where ΔCt = Ct (target mRNA) − Ct (control gene). All real-time assays were done in duplicate.

Fluorescence *In situ* Hybridization (FISH). FISH on head and neck cancer cell lines was done with the Locus Specific Identifier EGFR/Cep7 Dual Color FISH probe (Vysis, Inc., Downers Grove, IL), as per manufacturer's instructions. Briefly, cell pellets were fixed overnight in formalin, paraffin embedded, and sectioned on ChemMate slides at a 5-μm thickness. Unstained sections were dehydrated in an etomidate gradient, and 10 μL of probe mixture containing Locus Specific Identifier hybridization buffer and probe were applied to the slide followed by a sealed coverslip. Slides were placed in a prewarmed humidified box at 37 °C overnight. Next morning, slides were washed multiple times in prewarmed 0.4× SSC/0.3% NP40 wash buffer (Vysis, Inc.), air-dried, and counterstained for visualization with 4′,6-diamidino-2-phenylindole II. In each case, at least 30 cells were counted for both the egfr gene and chromosome 7 centromere signals with the Vysis, Inc., genetic workstation imaging software and the Nikon microscope at ×100 magnification with the oil immersion technique and recommended lens filters. The results of the FISH assay were reported as a ratio of the average copy number of the EGFR gene to that of the chromosome 7 centromere. Specimens with a signal ratio of <2.0 were designated as nonamplified and ≥2.0 as amplified, as described previously (28).

***egfr* Gene Silencing by siRNA.** Small interfering RNA (siRNA) specific for the *egfr* gene (Super Array, Frederick, MD) was used to induce *egfr* gene silencing in HN029 cells. HN029 cells were plated in a 24-well tissue culture plate at 5×10 (4) per well supplemented with 400 μL of RPMI 1640 growth medium and 10% fetal bovine serum. After 12 hours of incubation, the cells were transfected with siRNA and Lipofectamine 2000 (Invitrogen, Hercules, CA), according to the manufacture's protocol. Control consisted of HN029 cells in the presence of the transfection reagent without siRNA. The media was removed, and the cells were harvested 24 and 48 hours after the transfection to measure the amount of mRNA with quantitative real-time PCR analysis. The HN029 control cells were used as an internal control to compare the silencing of EGFR. The siRNA transfection was done in quadruplicate.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling. Quantification of apoptosis after siRNA transfection was assessed by the terminal deoxynucleotidyl transferase nick-end-labeling technique, with a kit commercially available (GuavaTUNEL kit, Guava Technologies, Hayward, CA).

Clinical Specimens. DNA was obtained from tumor and paired peripheral blood cells from 30 patients with head and neck cancer. Intron 1 polymorphism was determined as mentioned above. In addition, DNA was obtained from skin biopsies of 19 patients with advanced colorectal cancer who received daily treatment with 500 mg of gefitinib in a phase II study. Skin toxicity was evaluated in these subjects on a weekly basis and graded according to the National Cancer Institute Common Toxicity Criteria, v2.0 scale.

Data Analysis. The growth inhibition studies were done in triplicate and repeated three times. The average and 95% confidence interval were determined. The IC_{50} was determined for each cell line as the concentration of drug that inhibits 50% cell growth. The relative growth between cells with >35 or ≤35 sum of CA repeats in both alleles was compared with the Fisher's exact test. The relationship between mRNA, EGFR protein expression, and growth inhibition was explored with a linear regression analysis. The correlation between (CA)n in normal and tumor DNA was assessed by the Pearson correlation coefficient. A *t* student test was used to compare the mean number of CA repeats between subject with and without skin rash. Data were processed and analyzed with the SPSS statistical program (V.011).

RESULTS

***In vitro* Susceptibility to of Head and Neck Cancer Cell Lines to Erlotinib as a Function of (CA)n in Intron 1 of the *egfr* Gene.** We first determined the susceptibility to erlotinib, a specific EGFR TK inhibitor, in a panel of 12 head and neck cancer cell lines and the epithelial A431 cell line with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. Table 1 summarizes the results of these studies. Five cell lines were considered resistant ($IC_{50} > 10$ μmol/L), whereas 8 cell lines, including A431, were considered susceptible (IC_{50s}, 0.1 to 1 μmol/L). We next determined the number of CA dinucleotides in the CA-SSR polymorphic region of the intron 1 with microsatellite PCR analysis to check for differences in the number of CA dinucleotide repeats in the different cell lines. A431 was used as a reference because this cell line is known to be susceptible to EGFR-targeted drugs, and the number of CA dinucleotide repeats has been reported to be 16 of 16 (26). The analysis of the response to the drug as a function of individual allelic length revealed a nonstatistically significant increased in the IC_{50} to erlotinib from an average of 5 μmol/L for alleles <20 and 10 μmol/L for alleles ≥ 20. Likewise, the relative growth under erlotinib 1 μmol/L increased from 46 +/− 28 to 72 +/− 32% for alleles < and ≥ 20, respectively. Four of five cell lines (80%) with sum of CA dinucleotide repeats in both alleles > 35 were found to be resistant ($IC_{50} > 10$ μmol/L), whereas seven of the eight cell lines with sum of CA repeats ≤ 35 were

Table 1 *Response of head and neck cancer cell lines to erlotinib*

Cell line	Genotype	Relative growth erlotinib (1 μmol/L)	IC_{50} (μmol/L)
HN011	16/20	17	0.25
HN019	16/20	97	23
HN028	16/16	85	25
Detroit	16/20	72	3
HN006	16/19	32	0.05
HN022	16/19	30	0.35
HN012	16/19	40	0.93
CCL30	16/21	95	7
CAL27	16/16	17	0.10
HEP2	17/20	80	20
HN029	16/16	20	0.20
A431	16/16	27	0.30
HN013	16/19	50	0.40

sensitive with an IC_{50} of 0.1 to 1 μmol/L ($P = 0.03$). The relative growth of cells with long CA repeats (*i.e.,* >35) averaged 72 +/− 32% at the 1 μmol/L concentration in contrast to cells with ≤35 CA repeats, which had an averaged growth of 38 +/− 21% at the same concentration of erlotinib (Fig. 1*A*).

Relationship between Number of CA Dinucleotides and *egfr* mRNA and Protein Levels. We next determined the relationship between the number of CA dinucleotides and expression of the *egfr*. There was a statistically significant inverse correlation between the length of the CA dinucleotide repeats and the expression of the *EGFR* mRNA ($r = 0.54$, $P = 0.05$). Likewise, cells with longer alleles had lower levels of protein expression, albeit not statistically significant ($r = 0.48$, $P = 0.09$; Fig. 1*B*). To rule out the possibility that these results were due to amplification of the *EGFR* gene, a known mechanism of overexpression of the EGFR protein, we performed FISH in the panel of cell lines (29). None of the 12 head and neck cancer cell lines had amplification of the *egfr* gene by FISH assay (mean signal ratio EGFR/Cep7 = 1.17; range, 0.99 to 1.78; median = 1.06). A431 showed amplification of the *egfr* gene (signal ratio EGFR/Cep7 = 2.66) as described previously (29).

siRNA-mediated Reduction of EGFR Expression Results in Resistance to Erlotinib. On the basis of the above results, we next investigated whether specifically reducing the levels of EGFR expression in sensitive cell lines with a short number of CA dinucleotide repeats influenced the *in vitro* sensitivity to erlotinib. To this end, we silenced the expression of EGFR with siRNAs (30) The HN029 cell line was chosen for RNA interference studies because it expresses high levels of EGFR protein, has a 16/16 genotype, and is sensitive to

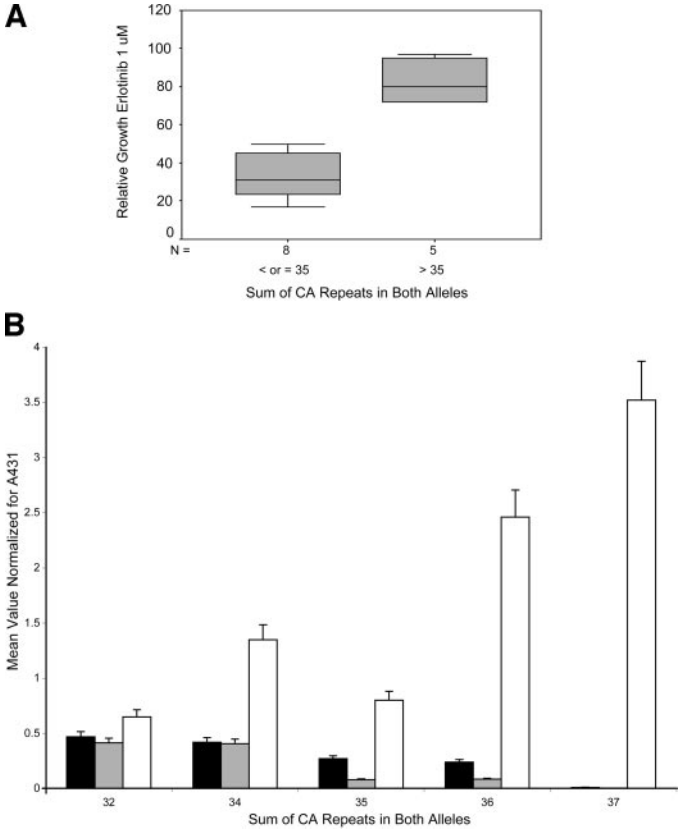

Fig. 1. *A*, box plot graph of relative growth in cells with long (>35 CA repeats) and short (≤35 CA repeats) intron 1 alleles when exposed to 1 μmol/L concentration of erlotinib. *B*, graphical illustration of the influence of the length of CA repeat allele on egfr protein expression, *egfr* mRNA levels, and growth inhibition after treatment with erlotinib at 1 μmol/L. Mean values with the corresponding SDs are showed. ■, EGFR; ▨, mRNA; □, relative growth.

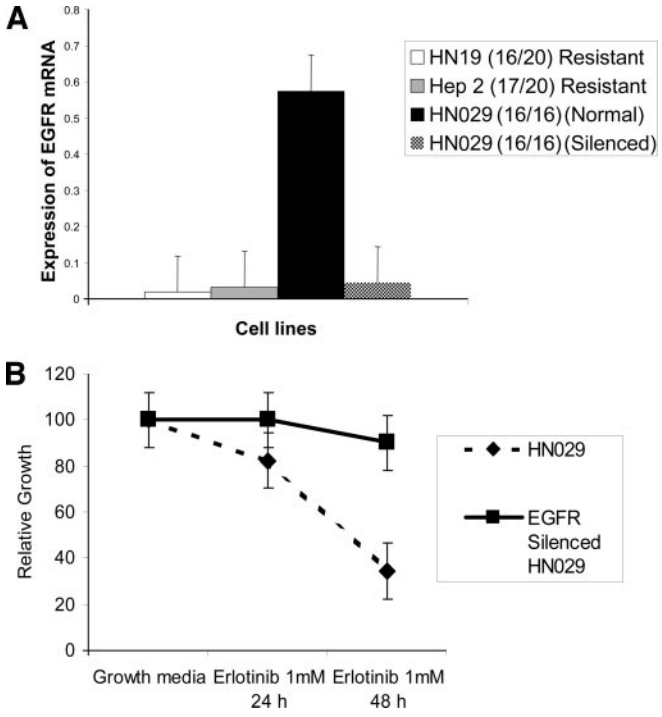

Fig. 2. *A*. siRNA directed against *EGFR* reduces mRNA levels. *Bars* represent *EGFR* mRNA/*GADPH* mRNA ratio after 48 hours in HN029 cell line (wild type), HN029 after silencing *EGFR* with siRNA, HN019, and Hep-2 cell lines. *B*, relative growth inhibition (response) to treatment with erlotinib. *Lines* represent relative growth after 24 and 48 hours of treatment with erlotinib 1 μmol/L in HN029 (wild type) and in the silenced cell line. After 48 hours of treatment, erlotinib inhibited relative growth in 66% in the original cell lines, whereas it only resulted in 10% of relative growth in the silenced one.

Table 2 *Response of HN029 wild-type and siRNA-modified cells to erlotinib*

	EGFR mRNA/GADPH	Relative growth (%)	Apoptosis (%)
HN029 erlotinib 1 μmol/Lм 48 h	0.57	33 (±1.9)	30 (±0.7)
siRNA EGFR HN029 erlotinib 1 μmol/L 48 h	0.04	90 (±2.0)	2 (±0.7)

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

treatment with erlotinib. After transfection with Lipofectamine2000 and the siRNA, quantitative reverse transcription-PCR was done to measure its efficiency. siRNA inhibited endogenous *egfr* mRNA by >95% of the transfected cells 48 hours after transfection, resulting in levels of EGFR similar to cells with longer CA dinucleotide repeats that are resistant to erlotinib (Fig. 2*A*). Once transfection was achieved successfully, we addressed whether the silencing of the *egfr* mRNA affected the susceptibility to erlotinib in HN029 cell line by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (Fig. 2*B*). Transfected cells showed no inhibition in their growth after 24 hours of treatment with erlotinib at 1 μmol/L and an inhibition of 10% 48 hours after treatment. In the control nontransfected cells, growth was inhibited 17% at 24 hours and >65% after 48 hours. Finally, to investigate the involvement of apoptotic cell death in these observed effects, we did a terminal deoxynucleotidyl transferase nick-end-labeling assay. After 48 hours of treatment with erlotinib, transfected cells showed 2% of apoptosis, whereas 30% of apoptotic death was observed in nontransfected cells. These results, therefore, show that inhibition of the *egfr* mRNA expression induces resistance to erlotinib (Table 2).

Number of CA Dinucleotide Repeats Is Identical in Tumor and Normal Tissue DNA. We next tested whether this region is conserved between normal and tumor tissues from the same individual.

To this end, we compared the number of CA dinucleotide repeats in matched DNA samples obtained from tumor and peripheral blood mononuclear cells from 30 subjects with advanced squamous cell carcinoma of the head and neck. We observed a perfect correlation ($r = 1$) in the (CA)n between paired samples of the same individual (data not shown), supporting the notion that this region is not commonly somatically mutated in the process of tumor development.

Number of CA Dinucleotide Repeats Is Associated with Skin Toxicity in Patients Treated with Gefitinib. The observation that the number of CA dinucleotide repeats is associated with response to EGFR inhibitors and that it is conserved region between normal and tumor tissues led to the hypothesis that the (CA)n is related to the occurrence of skin toxicity in patients treated with this drug. We determined the (CA)n in 19 patients with colon cancer treated with 500 mg oral daily doses of gefitinib in a phase II study. Overall, 68% of the patients developed the classic EGFR inhibitor induced acne-like rash. The allelic distribution in this group of patients was 14 (2.6%), 16 (47.4%), 17 (7.9%), 18 (18.4%), 20 (13.2%), and 21 (10.5%). The mean allelic length was 17 +/− 1.7 in subjects who developed rash *versus* 18.2 +/− 2 in this who did not ($P = 0.07$). The analysis of rash as a function of alleles revealed that lower frequency of rashes with longer alleles that decreased from 76% for alleles < 20 to 44% for alleles $\geq 20\%$ ($P = 0.09$). In the combined analysis by allelic sum, the mean sum of alleles was 34 +/− 2.6 in subjects who developed rash and 36.5 +/− 2.8 in those who did not ($P = 0.09$). Eighty-four percent of patients with sum of alleles of ≤ 35 developed a rash, whereas only 33% of those with sum of alleles > 35 did ($P = 0.04$).

DISCUSSION

This study explored the relationship between a CA-SSR polymorphism in the intron 1 of the *egfr* gene and the response to EGFR TK inhibitor in a panel of squamous cell carcinoma of the head and neck cell lines and in a clinical trial. The results show a correlation between the number of CA dinucleotide repeats and levels of EGFR mRNA and protein and the response to erlotinib. Phenotypic manipulations by RNA interference to decrease the levels of mRNA from susceptible cell lines with a short number of CA dinucleotide repeats to levels similar to that of resistant cells altered the response to the drug confirming the specificity of the findings. Subjects with a short number of CA dinucleotide repeats had higher incidence of rash when treated with one of these drugs. Because the number of (CA)n is equivalent in matched normal and tumor DNA in squamous cell carcinoma of the head and neck, this may offer a marker for subjects who experience skin toxicity when treated with these drugs have better outcome. These results have several biological as well as clinical implications.

The most obvious mechanism by which the CA-SSR is associated with response to EGFR TK inhibitors is by affecting the levels of the EGFR. The results of this study are in perfect agreement with prior studies indicating a an inverse correlation between the number of CA dinucleotides in the intron 1 region and the transcription of the gene and protein expression (24, 25, 31). These findings contrast with the lack of correlation found in clinical trials between expression of the EGFR and activity of EGFR TK inhibitors (6, 7, 15) The reasons for this discrepancy are not known but could be related to methodological issues regarding the use of non quantitative and poorly validated immunohistochemistry methods. In fact, studies have failed to show a correlation between number of CA dinucleotide repeats and EGFR protein expression measured by immunohistochemistry in colorectal cancer (32). In this regard, it is important to mention that studies measuring mRNA levels by RT-PCR from microdissected tumor tissue may be a predictive marker for response of metastatic colorectal tumors to therapy with EGFR inhibitors (33). This finding is fully supported by the data from this study. We found a better correlation between mRNA expression as opposed to protein expression and response to the drugs. The siRNA experiments additionally support the notion that expression of the target is related to the response to these agents. Overall, these data would support the notion that the expression of the target is important for the response to these agents and that this topic should still be additionally investigated with other methods such as reverse transcription-PCR before it is concluded that expression of EGFR is not relevant.

An interesting observation in population studies is that the CA-SSR polymorphism has interethnic variation. Of particular interest is the finding that subjects of Asian ethnicity have higher frequency of longer alleles. Sixty-three percent of individuals of Asian origin had alleles with 20 CA dinucleotide in contrast to 21% for individuals of Caucasian origin. Likewise, the frequency of allele 16 was most common in Caucasians (43%) and African Americans (42%) as opposed to Asians (17%; ref. 34). These results have been confirmed in a second study in patients with breast cancer from Japan and Germany that showed a higher frequency of alleles containing 19 or longer CA dinucleotides in Japanese patients, which was associated with lower levels of EGFR expression (31). These data, in conjunction with our data, would predict that the overall response to EGFR TK inhibitors would be lower in Japanese subjects. Studies conducted this far, however, show the opposite. Studies in NSCLC have shown that Japanese patients are in fact more susceptible to these drugs that patients from Western origin (6, 7). There are, however, two recent studies that may explain this apparent discrepancy. First, Japanese patients with NSCLC have a higher incidence of mutations in the exons coding the TK domain of the *EGFR* gene, which is the most important determinant of response to these agents in NSCLC patients (13, 14). This factor explains the higher response rata in Japanese patients. It should be noted, however, that thus far these mutations have not been found in diseases other than NSCLC. In addition, it is not know if Japanese patients with other tumor types have higher response to EGFR TK inhibitors. A second important observation is the finding of allelic imbalance restricted to the CA-SSR I region in 55% of Japanese patients with breast cancer, which is associated with amplification of that area of the gene and higher content of EGFR. A separate study found amplifications of the CA-SSR I in nonmalignant breast cancer tumors and to be associated with poor prognosis (31, 35). This mechanism appears to overcome the transcriptional limitations imposed by long alleles resulting in tumors with high content of EGFR that could be more susceptible to the agents. This factor could indeed explain why cells such as HN011, which have a 16/20 genotype, is in fact susceptible to the drug. Overall, these finding would suggest that there are different mechanisms by which germ-line or acquired genetic alterations in the *EGFR* gene can influence the response to these drugs, including kinase domain mutations, number of CA dinucleotide repeats, amplifications of the CA-SSR I region in addition to other such as amplification of the full gene, and vIII mutations frequently observed in brain tumors (36). How these different alterations are related to each other and which specific weight and implications have with regards to the response to the drugs would need to be the focus of future studies. Furthermore, it is possible that specific alleles in the germ-line CA-SSR mark different haplotypes of untested functional relevance. In fact, the *EGFR* gene has several single sequence and single nucleotide polymorphisms in the same intron and throughout the gene that could be in association and linked to the response to these agents.

One of the main reasons why we initiated these studies was to find an explanation for the observed association between skin toxicity and the response to EGFR inhibitors. Studies conducted with small mol-

ecules, as well as with monoclonal antibodies, have consistently shown an association between the development of rash and the response to this class of drugs measured by both response rate and overall survival (8, 11, 15, 37). The underlying reason for this association is not known. One reason could be pharmacological: there is a linear dose-response relationship in that subjects with higher plasma concentration of the drug develop more toxicity, as well as beneficial effects. It is clear from early phase I studies that the occurrence of cutaneous toxicity is dose-related, but pharmacological studies have not shown a relationship between plasma concentration of total drug and the development of rash for drugs in which this has been studied (15, 38). In addition, within the range of doses explored, there is not an indication that higher doses are associated with improved efficacy (6, 7). An alternative explanation is genetics: a genetic trait shared between normal and tumor tissue that is linked to the response to the drugs. The data from this study supports this hypothesis in that the CA-SSR is equal between normal and tumor tissues and is linked to the response to the agents. The implication of these data are that the CA-SSR should be explored as a determinant of outcome and that these measurements can be done in genomic DNA. From a practical perspective, assessment of intron 1 number of CA dinucleotide repeats as a predictor clinical outcome is very attractive because it can be easily measured in normal tissues (blood cells and skin), is a constant feature that does not change over time, and is technically simple, objective, easily exportable, and fully quantitative. It is important to note, however, that this measurement is unlikely to be the only predictor because tumors have another somatic genetic alterations such CA-SSR I amplification that can compensate the deficient transcription activity of long alleles.

In conclusion, the data presented here suggest a significant correlation between polymorphic variations in the intron 1 of the *EGFR* and response to the EGFR inhibitors. These data warrant additional large-scale clinical studies to validate and integrate *egfr* genotyping as a marker for the selection of patients to be treated with anti-EGFR–targeted therapies.

REFERENCES

1. Davies RL, Grosse VA, Kucherlapati R, et al. Genetic analysis of epidermal growth factor action: assignment of human epidermal growth factor receptor gene to chromosome 7. Proc Natl Acad Sci USA 1980;77:4188–92.
2. Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol Ther 1999;82:241–50.
3. Lacroix H, Iglehart JD, Skinner MA, Kraus MH. Overexpression of erbB-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. Oncogene 1989;4: 145–51.
4. Klijn JG, Berns PM, Schmitz PI, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr Rev 1992;13:3–17.
5. Veale D, Kerr N, Gibson GJ, Kelly PJ, Harris AL. The relationship of quantitative epidermal growth factor receptor expression in non-small cell lung cancer to long term survival. Br J Cancer 1993;68:162–5.
6. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non–small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
7. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. J Am Med Assoc 2003;290:2149–58.
8. Cohen EE, Rosen F, Stadler WM, et al. Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol 2003;21: 1980–7.
9. Saltz L, Meropol NJ, Loehrer PJ, et al. Single agent IMC-C225 (Erbitux) has activity in CPT-11-refractory colorectal cancer that expresses the epidermal growth factor receptor (EGFR). Proc Am Soc Clin Oncol 2002;21:127.
10. Finkler N, Gordon A, Crozier M, et al. Phase II evaluation of OSI-774, a potent oral antagonist of the EGFR-TK in patients with advanced ovarian carcinoma (Abst #831). Proc Am Soc Clin Oncol 2001;20:208.
11. Perez-Soler R, Chachoua A, Hammond L, et al. Determinants of tumor response and survival with erlotinib in patients with non-small cell lung cancer. J Clin Oncol 2004;22:3238–47.
12. Motzer RJ, Amato R, Todd M, et al. Phase II trial of antiepidermal growth factor receptor antibody C225 in patients with advanced renal carcinoma. Investig New Drugs 2003;21:99–101.
13. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 2004 May 20;350:2129–39.
14. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science (Wash. DC)304:1497–500.
15. Soulieres D, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. J Clin Oncol 2004;22:77–85.
16. Saltz L, Rubin M, Hochter H, et al. Acne-like rash predicts response in patients treated with Cetuximab (IMC-C225) plus irinotecan (CPT-11) in CPT-11 refractory colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFR). Proc Am Soc Clin Oncol 2003;22:204.
17. Lima JJ, Thomason DB, Mohamed MH, Eberle LV, Self TH, Johnson JA. Impact of genetic polymorphisms of the beta2-adrenergic receptor on albuterol bronchodilator pharmacodynamics. Clin Pharmacol Ther 1999;65:519–25.
18. Yoshida H, Mitarai T, Kawamura T, et al. Role of the deletion of polymorphism of the angiotensin converting enzyme gene in the progression and therapeutic responsiveness of IgA nephropathy. J Clin Investig 1995;96:2162–9.
19. Henrion D, Amant C, Benessiano J, et al. Angiotensin II type 1 receptor gene polymorphism is associated with an increased vascular reactivity in the human mammary artery in vitro. J Vasc Res 1998;35:356–62.
20. Benetos A, Cambien F, Gautier S, et al. Influence of the angiotensin II type 1 receptor gene polymorphism on the effects of perindopril and nitrendipine on arterial stiffness in hypertensive individuals. Hypertension 1996;28:1081–4.
21. van Essen CG, Rensma PL, de Zeeuw D, et al. Association between angiotensin-converting-enzyme gene polymorphism and failure of renoprotective therapy. Lancet 1996;347:94–5.
22. Arranz M, Collier D, Sodhi M, et al. Association between clozapine response and allelic variation in 5-HT2A receptor gene. Lancet 1995;346:281–2.
23. Chrysogelos SA. Chromatin structure of the EGFR gene suggests a role for intron 1 sequences in its regulation in breast cancer cells. Nucleic Acids Res 1993;21: 5736–41.
24. Buerger H, Gebhardt F, Schmidt H, et al. Length and loss of heterozygosity of an intron 1 polymorphic sequence of egfr is related to cytogenetic alterations and epithelial growth factor receptor expression. Cancer Res 2000;60:854–7.
25. Gebhardt F, Zanker KS, Brandt B, et al. Modulation of epidermal growth factor receptor gene transcription by a polymorphic dinucleotide repeat in intron 1. J Biol Chem 1999;274:13176–80.
26. Haley JD, Waterfield MD. Contributory effects of de novo transcription and premature transcript termination in the regulation of human epidermal growth factor receptor proto-oncogene RNA synthesis. J Biol Chem 1991;266:1746–53.
27. Layfield LJ, Bernard PS, Goldstein NS. Color multiplex polymerase chain reaction for quantitative analysis of epidermal growth factor receptor genes in colorectal adenocarcinoma. J Surg Oncol 2003;83:227–31.
28. Maitra A, Wanzer D, Weinberg AG, Ashfaq R. Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. Cancer (Phila.) 2001;92:677–83.
29. Merlino GT, Ishii S, Whang-Peng J, et al. Structure and localization of genes encoding aberrant and normal epidermal growth factor receptor RNAs from A431 human carcinoma cells. Mol Cell Biol 1985;5:1722–34.
30. Bass BL. Double-stranded RNA as a template for gene silencing. Cell 2000;101: 235–8.
31. Buerger H, Packeisen J, Boecker A. Allelic length of a CA dinucleotide repeat in the egfr gene correlates with the frequency of amplifications of this sequence: first results of an inter-ethnic breast cancer study. J Pathol 2004;203:545–50.
32. McKay JA, Murray LJ, Curran S, et al. Evaluation of the epidermal growth factor receptor (EGFR) in colorectal tumours and lymph node metastases. Eur J Cancer 2002;38:2258–64.
33. Stoehlmacher JBJ, Shirota Y, Park DJ, et al. Association between mRNA expression level of epidermal growth factor receptor (EGFR), immunohistochemistry (IHC), and response to the EGFR inhibitor C225 in advanced colorectal carcinoma (Abst #356). Proc Am Soc Clin Oncol Annu Meet 2001.
34. Liu W, Innocenti F, Chen P, Das S, Cook EH Jr, Ratain MJ. Interethnic difference in the allelic distribution of human epidermal growth factor receptor intron 1 polymorphism. Clin Cancer Res 2003 Mar;9:1009–12.
35. Tidow N, Boecker A, Schmidt H, et al. Distinct amplification of an untranslated regulatory sequence in the egfr gene contributes to early steps in breast cancer development. Cancer Res 2003;63:1172–8.
36. Rich JN, Reardon DA, Peery T, et al. Phase II trial of gefitinib in recurrent glioblastoma. J Clin Oncol 2004;22:133–42.
37. Saltz LB, Meropol NJ, Loehrer PJ Sr, Needle MN, Kopit J, Mayer RJ. Phase II trial of Cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 2004;22:1201–8. Epub 2004 Mar 1.
38. Hidalgo M, Siu LL, Nemunaitis J, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. J Clin Oncol 2001;19:3267–79.