Appendix

Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity.

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Toxicity Assessment and Grading

Toxicity assessment was performed at each study visit by study investigators. All symptomatic and objective toxicities and adverse events were recorded, assigned a relation to study drug, and graded using the National Cancer Institute Common Toxicity Criteria version 2.0

Genotyping of Genetic Polymorphisms

Blood DNA was extracted by using PUREGENE DNA Purification Kit (Gentra Systems, Inc, Minneapolis, MN). Four polymorphisms (-216G/T, -191C/A, intron 1(CA)n, and 497G/A) in the EGFR gene, CYP3A4*1B, CYP3A5*3 and six polymorphisms (-15994G/A, -15622C/T, intron 1 16702G/A, intron 1 1143C/T, 421C/A and 34G/A) in the ABCG2 gene were genotyped. Polymerase chain reaction (PCR) was performed to amplify the DNA sequences containing the polymorphisms of interest. Genotyping related DNA sequences and annealing temperatures are listed in Appendix Table A2. For -191C/A and -216G/T, PCRs were set up in a 40-μL volume containing 3 mM MgCl2, 1×Q-solution (Qiagen, Santa Clarita, CA), 100 μM each of dNTP, 125 nM of forward and reverse primers, 1 unit Hotstart Taq DNA polymerase (Qiagen), and 25 ng of DNA. Reactions were denatured initially at 98°C for 10 minutes and then cycled 35 times at 98°C for 15 seconds, annealing at 62°C for 15 seconds and 72°C for 20 seconds. PCR products were then purified and directly sequenced as previously described.1 For other polymorphisms, PCRs were performed in a 15-μL volume containing 125 nM of each primer for the two PCR amplicons, 30 ng of genomic DNA, 2.5 mM of MgCl2, 100 μM of each dNTP, and 0.375 U of AmpliTa Qgold polymerase (Applied Biosystems, Foster City, CA) in the buffer provided by the manufacturer. Duplex PCR was used to amplify the ABCG2 polymorphisms. Genotyping of the EGFR intron 1 (CA)n polymorphism was performed as previously described.2 Other polymorphisms, EGFR 497G/A, CYP3A4*1B, CYP3A5*3, and ABCG2 polymorphisms were genotyped using single-base extension and denaturing high-performance liquid chromatography (DHPLC). Briefly, amplified PCR products were purified by treatment with shrimp alkaline phosphatase (Roche, Neuilly sur Seine, France) and exonuclease I (USB) at 37°C for 45 minutes before the SBE reaction. SBE reactions were carried out in 12.6 μL containing 1 μM of each SBE primer, 250 μM each of four ddNTPs, 7.2 μL of purified PCR product and 1.5 U of ThermoSequenase (Amersham Pharmacia Biotech) in 1× Reaction buffer provided by the manufacturer. Reactions were run in a 9600 thermal cycler (Applied Biosystems) under the following conditions: 96°C for 2 min,

followed by 60 cycles of 96°C for 30 s, 55°C for 30 s and 60°C for 30 s. Wave 3500HT DHPLC system (Transgenomic Inc.) was used for separating SBE products. Before run on DHPLC, the samples were denatured at 96°C for 4 minutes and held at 4°C. For analyzing SBE on Wave DHPLC, 8 μL of SBE products of each sample was injected. We used “Mutation Detection” application type as a template, selected “Normal” clean as clean type (ie, 100% buffer B clean off step after each injection), and manually set the following variables for this application: The flow rate was set at 1.5 mL/min by using an high-throughput column, oven temperature was set at 70°C, and the gradient used for elution of the SBE products was from 24% to 36.5% buffer B over 2.5 minutes (buffer B contains 25% acetonitrile). The extended products were eluted in the order of C<G<T<A dependent on the hydrophobicity differences of the four bases. The known genotype controls were included in each run.

Haplotype and Diplotype Estimation

The haplotypes between EGFR -216G/T and -191C/A polymorphisms, between CYP3A4*1B and CYP3A5*3, and between ABCG2 intron 1 polymorphisms 1143C/T and −15622C/T were predicted, and the diplotypes for each sample were assigned by using Phase 2.0 program (https://innateimmunity.net/IIPGA2/Bioinformatics/Phase/phase_run). A probability cut-off of ≥ 0.9 was used for diplotype assignment.

Erlotinib Pharmacokinetic Analysis

Blood samples for pharmacokinetic analysis were collected into sodium heparinized vacutainer tubes on days 1, 15, and 29 of cycle 1. On day 1, samples were drawn before the first treatment dose and 0.5, 1, 2, 4, and 6 hours after treatment initiation. Blood samples were also collected before treatment on days 15 and 29 of the first cycle. Briefly, plasma aliquots (500 μL) were vortexed and centrifuged (650 rcf, 10 minutes, 4°C) after addition of 80 μL of internal standard (3.4 μg/mL of chloroquine) and 5 mL of methyl t-butyl ether. After centrifugation, the analytes were extracted by acidification with 1.2 mL of 5% hydrochloric acid and back-extracted using 1.2 mL of 1 N sodium hydroxide and 5 mL of methyl t-butyl ether. The supernatants were then evaporated to dryness using nitrogen gas (37°C) and reconstituted in 250 μL of mobile phase, and 200 μL aliquots were injected into the high-performance liquid chromatography (Hitachi High Technologies, San Jose, CA). The high-performance liquid chromatography conditions were identical to those published previously,3 with the only difference being that our mobile phase composition was 32/68 (volume/volume) acetonitrile/50 mM of potassium phosphate buffer containing 0.2% triethylamine (pH 4.8). Under our conditions, the retention times of chloroquine and OSI-774 were 2 and 18 minutes, respectively. Erlotinib concentrations were calculated using a standard curve (range, 15.3 to 4103.3 ng/mL). Samples with concentrations that fell outside of the range of the standard curve were reanalyzed by diluting with blank plasma before extraction, and the final concentration values were determined after applying the appropriate dilution factor. Intra-assay reproducibility (% CV = 1.1 to 10.0) and accuracy (range, 94.6 to 107.3) were determined by performing three measurements of the same seven standards on the same day. Inter-assay reproducibility (% CV = 4.5 to 11.2) and accuracy (range,

96.3 to 104.5) were determined by assays of the same seven standards in triplicate on 3 days.

Statistics and Data Analysis

NONMEM software (version V4; GloboMax LLC, Hanover, MD) was used for the pharmacokinetic data analyses. Dose, erlotinib concentration, and patient characteristics were fit simultaneously. Covariate models were selected based on the following selection criteria: a reduction of the objective function value (OFV) ≥ 3.84 units ($P \leq .05$, df = 1) for forward inclusion of a covariate in the model and a reduction of OFV ≤ 6.64 units ($P \leq .01$, df = 1) for backward elimination of a covariate from the model; physiologic relevance; decrease in the interpatient variability; and improvement in the diagnostic plots. The first-order conditional estimation method with interaction was used for the model development.

Individual exposures (area under the curve [AUC], maximum concentration [Cmax], and trough level [Ctrough] at steady-state) were estimated from the final population pharmacokinetic model by use of equations (1), (2), and (3), yielding a one-compartment model with first-order absorption and elimination. The patient characteristics considered for the model included age, height, weight, hematocrit, creatinine, albumin, and total bilirubin. Where AUC is the area under the drug concentration-time curve at steady-state, F is the bioavailability of drug, CL is the total drug clearance, CSS (t) is the drug concentration at steady state at time (t), ka is the absorption rate constant, V is the volume of distribution, k is the elimination rate constant, τ is the dosing interval, and tSS, max is the time at the maximum of concentration.

Logistic regression was performed to examine the association between PK parameters (AUC, Cmax, and Ctrough) and toxicity (cycle 1 rash and diarrhea). In these and all subsequent analyses, two sets of comparisons were performed, one dichotomizing toxicity as grade 0 versus grade 1 or worse (ie, none v any toxicity) and a second categorizing toxicity as grade 0 or 1 versus grade 2 or worse. t tests and analysis of variance were performed to evaluate the association between the various polymorphisms and PK parameters. As the modes of inheritance were either unknown or not clearly identifiable from the plotted data, four types of analyses were generally conducted, an analysis of variance on two degrees of freedom (df) to test the null hypothesis of equal mean levels of the PK parameters across the three genotypes, followed by t tests and linear regression analysis for the evaluation of dominant, recessive, and additive models. For example, for the hypothetical variant A>B, we have genotypes A/A, A/B, and B/B. In the additive model, the independent variable would be coded as follows: A/A=0, A/B=1, B/B=2. The dominant model would be coded as follows: A/A=0, A/B=1, B/B=1. The recessive model would be coded as follows: A/A=0, A/B=0, B/B=1

Fisher exact tests were used to analyze the association between genetic polymorphisms and toxicity. For the EGFR intron 1 (CA)n polymorphism, we examined the association between the number of CA repeats and toxicity as follows. The number of CA repeats on a given allele ranged from 14 to 22, with one additional allele having

25 repeats. We prospectively hypothesized that patients with alleles ≤ 16 repeats would have a different phenotype from those with longer repeats. We therefore classified patients as “s/s” if the number of repeats was ≤ 16 on both alleles, “l/l” if the number of repeats was > 16 on both alleles, and “s/l” if the number of repeats on one allele was ≤ 16 and the number for the other allele was > 16. However, we also considered additional cut points, r, of 17, 18, 19, and 20. The data were analyzed using Fisher's exact test as well as Armitage's test for a linear trend in proportions.5

P values between .05 and .10 were regarded as marginally suggestive of an association (boldfaced), whereas P < .05 was considered statistically significant (boldfaced and italicized). Only significant or marginally significant P values are shown in the tables, with other results denoted as not significant (“NS”). Note, however, that for the CA repeat analysis, because five different cut points were considered, a Bonferroni correction would require P < .05/5= .01 for statistical significance.