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Epidermal growth factor receptor polymorphisms and risk for toxicity in paediatric patients treated with gefitinib

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ABSTRACT

Purpose: To investigate associations between germline genetic variations in the epidermal growth factor receptor (EGFR) and toxicity in paediatric patients treated with gefitinib.

Patients and methods: Gefitinib treatment and toxicity data from five paediatric clinical trials were combined. *EGFR* genotypes evaluated included –191C>A, –216G>T, Arg497Lys and intron 1 CA sequence repeat number. The genetic variations were evaluated for associations with grade one or greater rash or diarrhoea during the first course of treatment.

Results: The analysis included 110 patients, 60 (55%) with grade one or greater rash and 47 (43%) with grade one or greater diarrhoea. Among patients with the –216 GG ($n = 51$), GT ($n = 41$) and TT ($n = 16$) genotypes, grade one or greater rash occurred in 52.9%, 46.3% and 87.5% of patients ($p = 0.003$, recessive model), respectively. Diarrhoea occurred in 27.5%, 58.5% and 43.8% of patients with respective GG, GT and TT genotypes ($p = 0.004$, dominant model). The –191C>A, intron 1 CA repeat number and Arg497Lys genotypes were not significantly associated with either rash or diarrhoea. EGFR –216 and –191 polymorphisms were in linkage disequilibrium ($D' = 0.66$, $p = 0.01$). The haplotype (–191C, –216T) was associated with increased risk for rash ($p = 0.049$), but was not more predictive of rash than the single –216 polymorphism.

Conclusion: These findings indicate that *EGFR* –216G>T genotype is a predictive marker for the development of skin rash and diarrhoea in paediatric patients treated with gefitinib. Continued investigation of relationships between germline EGFR polymorphisms and the efficacy of EGFR inhibitors in paediatric patients is warranted.

1. Introduction

The human epidermal growth factor receptor (EGFR) plays an important and diverse role in cellular signalling with influences on cellular proliferation, apoptosis, angiogenesis and metastasis.[1] In cancer cells, activation of EGFR and subsequent tyrosine kinase phosphorylation of the intracellular domain leads to a series of intracellular signals, resulting in

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increased tumour cell growth, division and resistance to apoptosis.[2] Inhibition of EGFR intracellular signalling with 4-anilinoquinazoline derivatives (e.g. erlotinib and gefitinib), which function as EGFR tyrosine kinase inhibitors (TKIs) has arisen as an effective anticancer treatment strategy.[3]

Despite multiple influences on cancer cell growth and division, inhibition of the EGFR pathway with TKIs only produces clinical responses in subgroups of patients.[4] Factors associated with sensitivity to EGFR TKIs include EGFR amplification and activating mutations influencing the ATP-binding pocket of the tyrosine kinase domain.[5–8] However, neither amplification nor mutational status is sufficient to completely explain clinical responses to EGFR TKIs. The efficacy of anti-EGFR therapy has also been associated with the intensity of skin rash, one of the major side-effects of EGFR-targeted drugs.[9] Germline genetic variation in the expression and function of the *EGFR* gene may, in part, predict the probability of systemic responses such as rash and diarrhoea in patients and may also influence disease response in patients treated with EGFR TKIs.[10–13]

The *EGFR* gene, located at 7p12.3-p.1, contains multiple polymorphic variants.[12] Several of these variants lead to alterations in *EGFR* expression and signalling. The *EGFR* –216G>T polymorphism is located in the promoter region and influences binding of Sp1, a transcription factor essential to *EGFR* expression.[2] The –216T allele increases promoter activity and expression of *EGFR*.[14] Expression is also influenced by the –191C>A polymorphism in the promoter region.[14] Intron 1 contains a dinucleotide CA repeat (CA[*n*]). The number of CA repeats is inversely related to *EGFR* expression, with the most common CA repeat number being 16.[15,16] An additional variation located at codon 497 (Arg497Lys) results in an amino acid change in the extracellular domain of EGFR with considerable effects on ligand binding.[17]

Recent data from adult trials revealed significant associations between germline *EGFR* genetic variations and responses to EGFR TKIs including toxicity and disease response to therapy.[10–13,18] Continuing investigations indicate a growing potential for EGFR TKIs in treating paediatric solid tumours.[19–21] However, the relationship between phenotypic responses to EGFR TKIs and germline genetic variation has not been evaluated in a paediatric population. The aim of this study was to analyse the association between *EGFR* genotypes (*EGFR* –216G>T, *EGFR* –191C>A, intron 1 CA(*n*) and Arg497Lys) and the common toxicities of rash and diarrhoea in paediatric patients treated with the EGFR TKI, gefitinib.

2. Patients and methods

2.1. Study design and treatment

Patients who consented to provide blood samples for pharmacogenetic analysis were included in this study if adequate DNA was available for *EGFR* genotyping, complete toxicity information was available from clinical trial data and at least one full course of gefitinib therapy was completed on one of the five clinical trials evaluating gefitinib in paediatric solid tumours (Table 1). These included three phase one trials in which gefitinib was administered concomitantly with irinotecan. In the other two studies, gefitinib was administered as a single agent. Toxicity and treatment data in this analysis were limited to the first course of treatment, 21 d for studies 1, 2 and 3 and 28 d for studies 3 and 4. St. Jude Children's Research Hospital Institutional Review Board approved the subsequent genotyping and retrospective collation and analysis of clinical trial data. Study personnel who were unaware of the genotyping results performed the chart review for toxicity.

2.2. Drug administration

Gefitinib was administered orally once daily to all patients. In two studies (Nos. 4 and 5) gefitinib was administered as a single agent continuously for 28-day cycles. In three studies (Nos. 1, 2 and 3), gefitinib was administered days 1 to 12 of 21-day cycles, while irinotecan was administered concomi-

Table 1 – Characteristics of clinical trials from which data were derived.

Study number	Disease	Gefitinib dosage	Gefitinib schedule	Irinotecan dosage
1 Phase I (NCT00186979)	Refractory solid tumours	112.5, 150 mg/m^2	Days 1–12	15, 20 mg/m^2[a]
2 Phase I (NCT00132158)	Refractory solid tumours	150 mg/m^2	Days 1–12	10, 15 mg/m^2[b]
3 Phase II (NCT00135135)	Advanced, high-risk neuroblastoma	112.5 mg/m^2	Days 1–12	15 mg/m^2
4 Phase I (NCT00040781)	Refractory solid tumours	150, 300, 400, 500 mg/m^2	Days 1–28	–
5 Phase I/II (NCT00042991)	Newly diagnosed brain stem tumours or incompletely resected supratentorial malignant gliomas	100–375 mg/m^2	Days 1–28	–

[a] Irinotecan was administered intravenously with the exception of one oral dose during the first course.
[b] Irinotecan was administered orally with the exception of one intravenous dose during the first course.

tantly on days 1–5 and days 8–12. All irinotecan-containing protocols stipulated that patients receive atropine at 0.01 mg/kg (0.4 mg) intravenously for symptoms of acute cholinergic syndrome and loperamide at the first indication of diarrhoea.

2.3. Evaluation of clinical toxicity

In three of the protocols (Nos. 1, 4 and 5), rash and diarrhoea were graded according to National Cancer Institute (NCI) Common Toxicity Criteria version 2.[22] For the two remaining protocols (Nos. 2 and 3) the NCI Common Terminology Criteria for Adverse Events version 3 was used.[23] Because the definitions for grade one or greater rash and grade one or greater diarrhoea are similar between these two versions of the NCI adverse event-grading criteria, toxicity data were imported as recorded in the clinical trial protocol databases.

2.4. EGFR genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells using standard molecular procedures, and 10 ng of DNA from each patient was used for genotyping. The *EGFR* Arg497Lys polymorphism was genotyped as described,[24] with minor modifications. Samples were initially denatured at 95 °C for 5 min, then denatured at 94 °C for 1 min, annealed at 59 °C for 30 s and extended 72 °C for 30 s for 35 cycles. A final extension was performed at 72 °C for 10 min. Amplified products underwent BstNI restriction enzyme digest (New England BioLabs, Beverly, MA) at 60 °C for 2 h. Digested products were analysed on 4% agarose E-Gels (Invitrogen, Carlsbad, CA). The *EGFR* intron 1 CA(*n*) repeat polymorphism was analysed with previously reported primers and methods.[10] Amplifications were performed in 15 mL reactions containing 10–20 ng template, TrueAllele PCR premix (Applied Biosystems, Foster City, CA) and 0.5 mM primers. The PCR profile consisted of 94 °C for 5 min, 35×(94 °C for 60s, 60 °C for 60s, 72 °C for 60s) and 72 °C for 10 min. PCR products were run on ABI 3730 DNA Analyzer using an internal size standard, Genescan 400HD (Applied Biosystems). Fragment size analysis was performed with GeneMapper 4.0 software (Applied Biosystems). The *EGFR* –191 and –216 polymorphisms were genotyped as described,[10] with minor modifications. Samples were initially denatured at 95 °C for 15 min, then denatured at 94 °C for 30 s, annealed at 54 °C for 30 s and extended at 72 °C for 45 s, for 35 cycles. A final extension was performed at 72 °C for 10 min.

2.5. Statistical methods

All analyses were performed using the statistical software R.[25] Linkage disequilibrium (LD) analysis was used to determine the association between different pairs of genetic variants. We used D' (scaled D) to quantify the LD. D' is between –1 and 1 and the absolute value for D' of 1 denotes complete LD, whereas a value of 0 denotes complete linkage equilibrium. We used haplo.em function in the haplo.stats package to determine the haplotype frequencies for *EGFR* –191C>A and *EGFR* –216G>T. The haplo.score function was used to determine the association of haplotypes with diarrhoea and skin toxicity. We used Fisher exact test to compare the incidence rate of diarrhoea and skin toxicity between homozygous T and G alleles of *EGFR* –216G<T. Severe grades of rash and diarrhoea were infrequent in all protocols evaluated, thus genotypes were tested for association with grades 1 and greater rash and diarrhoea. The association function in the SNPassoc package was used to determine the association of a single genetic marker with diarrhoea and skin toxicity under five different genetic models; codominant, dominant, recessive, overdominant and log-additive. For all analyses, p-values less than 0.05 were considered statistically significant and p-values > 0.05 but <0.1 considered marginally significant, p-values were not adjusted for multiple comparisons.

3. Results

3.1. Patients

A total of 110 patients with *EGFR* genotyping and complete treatment and toxicity data were included in this analysis. Patient characteristics and genotype frequencies are summarised in Table 2. The gefitinib dosages administered to patients ranged from 100 mg/m^2 to 500 mg/m^2 with a median of 250 mg/m^2. Among the 42 patients treated concomitantly with irinotecan, the median dosage of irinotecan was 15 mg/m^2 (range, 10–20 mg/m^2). Adequate DNA was available for genotyping *EGFR* –191C>A in 98 patients, *EGFR* –216G>T in 108 patients, intron 1 CA(*n*) in 109 patients and Arg497Lys in 106 patients.

3.2. Prevalence of rash and diarrhoea

Grade one or greater rash occurred in 60 patients (55%) and 47 patients (43%) had grade one or greater diarrhoea during the first course of treatment (Table 3). We evaluated protocol characteristics, including concomitant irinotecan in relation to the proportion of patients with rash and diarrhoea (Table 3). In patients receiving concomitant irinotecan ($n = 42$) and not receiving irinotecan ($n = 68$), grade one or greater rash occurred in 52.3% and 55.9% of patients, respectively ($p = 0.8$). Grade one or greater diarrhoea was significantly associated with concomitant irinotecan administration. Grade one or greater diarrhoea occurred 90.5% in patients receiving irinotecan and 13.2% in those not receiving irinotecan ($p < 0.001$). No significant association was noted between gefitinib dosage and rash ($p = 0.3$).

3.3. Genetic polymorphisms and haplotype analysis

EGFR –191 and –216 distributions were in linkage disequilibrium ($D' = 0.66$, $p = 0.01$). The estimated haplotype frequencies for *EGFR* –216/–191 were 11.5%, 54.6% and 32.2% for G/A, G/C and T/C, respectively. The haplotype *EGFR* –216T/–191C was associated with increased risk of rash ($p = 0.049$). There were no significant associations between any of the *EGFR* –216/–191 haplotypes and diarrhoea in both the entire evaluable population and the subset not exposed to irinotecan ($n = 68$).

Table 2 – Patient characteristics by clinical trial.

	No. 1 $n = 21$	No. 2 $n = 2$	No. 3 $n = 19$	No. 4 $n = 17$	No. 5 $n = 51$	Total $n = 110$
Median age, years (range)	11.1	16.5	3.3	12.4	7.3	7.4 (1.6–21)
Male	9		17	5	22	53
Female	12	2	2	12	29	57
Caucasian	14	2	12	13	37	79
Other	7	–	7	4	14	31
Median body surface area, m^2 (range)	1.26	1.68	0.63	1.23	1.07	1.02 (0.51–2.54)
EGFR-191						
CC	15	–	11	12	36	74
CA	2		3	4	13	22
AA	1				1	2
EGFR-216						
GG	6		9	9	27	51
GT	8	2	10	7	14	41
TT	6			1	9	16
EGFR-497						
Arg/Arg	10	1	13	10	28	62
Arg/Lys	7	1	6	6	19	39
Lys/Lys	1			1	3	5
Intron 1 CA(*n*) repeat total						
S ($\leqslant$35)	12	2	11	7	22	54
L (>35)	8		8	10	29	55

Table 3 – Observed grades of rash and diarrhoea by clinical trial.

Toxicity	No. 1 ($n = 21$) *n* (%)	No. 2 ($n = 2$) *n* (%)	No. 3 ($n = 19$) *n* (%)	No. 4 ($n = 17$) *n* (%)	No. 5 ($n = 51$) *n* (%)	Total ($n = 110$) *n* (%)
Rash (grade $\geqslant$ 1)	9 (43)	1 (50)	12 (63)	12 (71)	26 (51)	60 (55)
Diarrhoea (grade $\geqslant$ 1)	21 (100)	2 (100)	15 (79)	4 (24)	5 (10)	47 (43)
	Grade 1 (%)		Grade 2 (%)			Grade 3 (%)
Observed grades of rash and diarrhoea						
Rash	46 (41.8)		13 (11.8)			1 (0.9)
Diarrhoea	23 (20.9)		15 (13.6)			9 (8.2)

3.4. *EGFR –216G>T genotype*

Among patients with the –216 GG, GT and TT genotypes, grade one or greater rash occurred in 52.9%, 46.3% and 87.5% of patients ($p = 0.003$, recessive model), respectively (Table 4). Grade one or greater diarrhoea occurred in 58.5% of patients with a GT genotype compared to 27.5% of patients homozygous for the G allele ($p = 0.004$, dominant model). Grade one or greater diarrhoea occurred in 43.8% of patients homozygous for the T allele.

Because irinotecan is associated with toxicities (e.g. diarrhoea), we chose an additional analysis of this toxicity–genotype relationship in those patients that did not receive irinotecan. In the 68 patients without exposure to irinotecan, rash and diarrhoea occurred in 55.9% and 11.8% of patients, respectively. In this subset of patients, grade one or greater rash occurred in 55.6%, 47.6% and 80% of patients with respective –216 GG, GT and TT genotypes ($p = 0.09$, recessive model). Grade one or greater diarrhoea occurred in 5.6%, 23.8% and 10% of patients with respective –216 GG, GT and TT genotypes ($p = 0.08$, dominant model).

3.5. *EGFR –191C>A genotype*

No significant relationships were observed between the *EGFR* –191C>A polymorphisms and either rash or diarrhoea in the 98 patients with available genotypes. Among the patients homozygous for the C allele ($n = 74$), grade one or greater rash occurred in 59.5% of patients, compared to that in 50% with the A/C genotype ($n = 22$). The incidence of diarrhoea for these two groups was 40.5% and 31.8%, respectively. Two patients were homozygous for the A allele of which one experi-

Table 4 – Grade 1 or greater rash and diarrhoea by *EGFR* polymorphism.

Polymorphism	Genotype	*n*	Rash%	*p*-Value	Diarrhoea%	*p*-Value
Intron 1 CA(*n*)	Short (⩽35)	62	48.1	NS	36.3	NS
	Long (>35)	47	61.8		48.1	
216G>T	G/G	51	52.9	0.01[a]/0.003[b]	27.5	0.01[a]/0.004[c]
	T/G	41	46.3		58.5	
	T/T	16	87.5		43.8	
191C>A	C/C	74	59.5	NS	40.5	NS
	A/C	22	50		31.8	
	A/A	2	0		50	
Arg497Lys	Arg/Arg	62	48.3	0.07[c]	41.9	NS
	Lys/Arg	39	66.7		41	
	Lys/Lys	5	60		20	

[a] Codominant model.
[b] Recessive model.
[c] Dominant model.

enced grade one or greater diarrhoea and neither experienced rash.

3.6. *EGFR intron 1 CA(n)*

The *EGFR* intron 1 CA repeat number ranged from 14 to 21 repeats. The most common *EGFR* intron 1 CA(*n*) variant was 16 repeats. Of the 109 patients with intron 1 CA(*n*) data, 79 (72.4%) had at least one allele of 16 repeats. The most common genotypes were 16/16 (17.4%), 16/18 (16.5%) and 16/20 (14.7%). Grade one or greater rash occurred in 29 of 47 (61.8%) of patients with a long sequence repeat (total CA repeat in both alleles of greater than 35) and in 29 of 62 (48.1%) of the patients with a short tandem repeat ($p = 0.8$). Grade one or greater diarrhoea occurred in 36.3% of patients with a long tandem repeat and in 48.1% of patients with a short tandem repeat ($p = 0.6$).

3.7. *EGFR Arg497Lys genotype*

For Arg497Lys, the distribution of the genotypes was 62 (58.4%) Arg/Arg, 39 (36.8%) Arg/Lys and 5 (4.7%) Lys/Lys. Grade one or greater rash occurred in 48.3% of patients with the Arg/Arg genotype, 66.7% of patients with the Arg/Lys genotype and 60% of patients with the Lys/Lys genotype; differences were marginally significant ($p = 0.07$ in dominant model). Grade one or greater diarrhoea occurred in 41.9%, 41% and 20% of patients with the respective Arg/Arg, Arg/Lys and Lys/Lys genotypes, these differences were not statistically significant.

4. Discussion

Although the association between *EGFR* polymorphisms and phenotypic response to gefitinib has been reported in adults,[10–12] this is the first report of a similar relationship in paediatric patients. Paediatric patients homozygous for the –216T allele were more likely to experience rash and more likely to experience diarrhoea compared to those homozygous for the G allele. In addition, the *EGFR* –216/–191 haplotype T/C was significantly associated with rash; although, not more predictive than *EGFR* –216 alone. The *EGFR* –216G>T polymorphism is located in the promoter region with the –216T allele leading to an increase in promoter activity and expression of *EGFR*.[14] The increased expression of *EGFR* in cell lines has been associated with an increased sensitivity to EGFR inhibition and this interaction may account for the genotype-toxicity relationships we observed.[14,15]

Three of the protocols evaluated included gefitinib administered concomitantly with irinotecan, which is known to cause diarrhoea. A significant association was observed with diarrhoea and exposure to irinotecan ($p < 0.001$), which was a confounding variable in our analysis. Therefore, we also evaluated the *EGFR* –216G>T genotype for association with diarrhoea and rash in the subgroup of patients not exposed to irinotecan. Although not statistically significant, a similar trend of an increased prevalence of diarrhoea and rash with the –216T allele was found in this subgroup without exposure to irinotecan.

In prior investigations in adults, both *EGFR* –216G>T and intron 1 CA(*n*) were significantly related to phenotypic responses to gefitinib. Liu and colleagues evaluated the relation between clinical outcomes and germline *EGFR* genetic variation including *EGFR* –216G>T, *EGFR* –191C>A, intron 1 CA(*n*) and Arg497Lys.[12] Their analysis included 92 adult non-small cell lung cancer (NSCLC) patients treated with gefitinib. Similar to our results, only *EGFR* –216G>T was significantly associated with rash or diarrhoea. In addition, their analysis revealed that patients homozygous for short intron 1 CA(*n*) (CA repeats of 16 or fewer) and at least one T allele of *EGFR* –216G>T experienced greater progression-free survival. In their investigation there were no significant relationships between the genotype results for *EGFR* –191C>A and Arg497Lys and the clinical outcomes of response, progression-free survival, overall survival or toxicity (either rash or diarrhoea).

Another investigation reported by Gregorc and colleagues[10] evaluated the relation between *EGFR* –216G>T, –191C>A and Intron 1 CA(*n*) and clinical benefit in 170 adult NSCLC patients treated with gefitinib. Based on a strong linkage disequilibrium of the distribution of *EGFR* –216G>T and –191C>A in their population, the two genotypes were analysed as haplotypes. In their analysis, absence of the *EGFR**1

genotype (defined as –216G/–191C) was associated with a significant improvement (56% versus 34%, $p = 0.011$) in the rate of clinical benefit with gefitinib.

Our investigation has shown for the first time the importance of germline *EGFR* genetic variation in the development of rash during treatment with EGFR TKIs in a paediatric population. Obtaining a sufficient patient population for a useful analysis required data to be pooled from multiple clinical trials consisting of a wide range of solid tumours. This limited the ability to assess relationships between the genetic variants and measures of drug efficacy. Thus, the phenotypic responses investigated were the most frequently reported adverse events with gefitinib: rash and diarrhoea.

In this analysis, three of the genetic variants evaluated, *EGFR* –191C>A, intron 1 CA(*n*) and Arg497Lys were not significantly associated with the occurrence of either rash or diarrhoea. This finding is similar to those by Liu and colleagues[12] and Gregorc and colleagues.[10] In addition, an analysis conducted by Han and colleagues evaluated tumoural tissue for EGFR mutations and intron 1 CA(*n*) from either tumoural or normal tissue of 86 adult NSCLC patients in Korea.[26] Coinciding with our results, intron 1 CA(*n*) was not associated with skin rash. In a multivariate analysis adjusted for sensitising mutations in *EGFR* exons 18–21, a low CA repeat number ($\leqslant$37) was independently associated with an improvement in response rate and time to progression in patients treated with gefitinib.

Other investigations of *EGFR* Arg497Lys and intron 1 CA(*n*) genotypes have indicated their importance in *EGFR* signalling and as indicators of prognosis.[27–29] Although our results did not find statistically significant associations between *EGFR* Arg497Lys, intron 1 CA(*n*) or *EGFR* –191C>A genotypes and rash or diarrhoea, potential relationships could exist between these genotypes and gefitinib effectiveness, or disease prognosis, that this study could not identify.

Differences in the distribution of the genetic polymorphisms among different racial backgrounds may also play an important role in our findings. The majority (72%) of the patients in our study were classified as Caucasian. The variant forms of *EGFR* –216, –191 and the shorter intron 1 CA(*n*) genotypes occur more frequently in patients of Northern European descent compared to East Asians.[16,18] Nomura and colleagues found activating mutations of *EGFR* in greater numbers of patients with shorter intron 1 CA(*n*), specifically in patients of East Asian descent.[18] The relationship between somatic mutations in the *EGFR* gene and germline genotype indicates that assessment of both germline genotype and tumour-related changes in EGFR may improve the ability to predict disease response to EGFR TKIs.

Rash has been demonstrated to be significantly associated with improved disease responses to EGFR TKI therapy.[9] Our findings indicate that germline genetic data may be useful to identify patients with a greater likelihood to develop rash while on EGFR TKI therapy. As demonstrated recently, improving patient selection can improve the effectiveness of anti-EGFR TKI therapy and avoid unnecessary toxicity in patients who may not benefit.[30] Our data, although limited to adverse events, support the importance of germline genetic variation in predicting response to gefitinib in paediatric patients. Combining evaluations of germline *EGFR* genotype, particularly *EGFR* –216G>T, with evaluations of tumoural EGFR mutation and amplification status in future prospective investigations of EGFR TKIs has the potential to enhance patient selection for treatment and improve the understanding of mechanisms involved in EGFR regulation.

Conflict of interest statement

Drs. McKibbin, Zhao, Tagen, Allen, Geyer and Stewart have no relevant conflicts of interest to disclose. Drs. Daw, Furman and McGregor report receiving research funding from Astra Zeneca.

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