Association of Epidermal Growth Factor Receptor Activating Mutations with Low *ERCC1* Gene Expression in Non-small Cell Lung Cancer

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Introduction: Patients with non-small cell lung cancer (NSCLC) with cancers harboring activating mutations in the epidermal growth factor receptor (EGFR) show improved efficacy from EGFR tyrosine kinase inhibitors. Some clinical studies also suggest enhanced efficacy of platinum-based chemotherapy in patients with EGFR-mutant cancers. We investigated the relationship of EGFR mutation status and DNA repair capacity, as exemplified by excision repair cross-complementing 1 (*ERCC1*) gene expression, as a potential explanation for this observation.

Methods: Microdissected formalin-fixed paraffin-embedded tumors from 1207 patients with NSCLC were analyzed by real-time polymerase chain reaction for mRNA expression levels of ERCC1 and for EGFR mutation status by an allele-specific polymerase chain reaction assay.

Results: NSCLC subtype was adenocarcinoma (AC) in 712 patients, squamous in 175, and not otherwise specified or other in 320. EGFR activating mutations were detected in 183/1207 patients (15.2%). Median ERCC1 expression overall was 1.82 (range, 0.22–27.31) and was histology related: AC, median = 1.68 (0.22–11.33) and squamous, median = 2.42 (0.51–14.28) ($p < 0.001$). Using a previously defined reference level of <1.7, ERCC1 expression was categorized as low in 556 of 1207 patients (46.1%). The presence of EGFR mutations was highly associated with ERCC1 expression ($p < 0.001$). This association was retained when adjusting for AC histologic subtype ($p = 0.001$).

Conclusions: NSCLC specimens harboring EGFR activating mutations are more likely to express low ERCC1 mRNA levels. Whether these findings translate into enhanced clinical efficacy of EGFR-mutant cancers to platinum-based chemotherapy remains to be determined.

Key Words: EGFR mutations, ERCC1 expression.

(*J Thorac Oncol.* 2010;5: 1933–1938)

Platinum-based chemotherapy remains a standard of care for advanced stage non-small cell lung cancer (NSCLC), resulting in improved survival, symptom control, and superior quality of life compared with patients receiving best supportive care.[1–3] Nevertheless, initial reports describing dramatic responses in patients with chemotherapy-refractory NSCLC with cancers harboring activating mutations in the epidermal growth factor receptor (EGFR) to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib raised the question of whether chemotherapy should remain the therapeutic standard in this biomarker-defined subgroup.[4–7]

Subsequently, randomized clinical trials have been undertaken to compare the efficacy of EGFR TKIs versus platinum-based chemotherapy in the first-line therapy for advanced NSCLC patient populations that are either EGFR mutation positive or enriched for clinical-pathologic features favoring EGFR mutation, such as East Asian ethnicity, female gender, and never-smoking status.[8–10] Overall, these trials support the conclusion that EGFR TKIs are superior to initial chemotherapy in this biologically distinct NSCLC subgroup, especially with respect to response rate and progression-free survival (PFS). In one such trial, Iressa Pan-Asia Study (IPASS), in which gefitinib was compared with carboplatin-paclitaxel chemotherapy in a clinically and pathologically enriched NSCLC patient population, approximately 60% of patients with available tumor tissue (retrospectively tested) proved positive for EGFR activating mutations.[8] As anticipated, response rate and PFS in patients with EGFR mutations were markedly increased compared with chemotherapy. Nevertheless, efficacy in the chemotherapy arm was also surprisingly good in the EGFR-mutant

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Disclosure: David R. Gandara, MD, is a consultant for Amgen, AstraZeneca, Biedsix, Boehringer-Ingelheim, Genentech, Merck, Novartis, and Sanofi-Aventis and has received grants from BMS/ImClone, Genentech, Lilly, Merck, Novartic, and Pfizer. Primo N. Lara, Jr., MD, was paid an honoraium to speak at the genetics satellite conference/symposium at WCLC 2009 in San Francisco. Peter Grimminger, MD, is a consultant for Response Genetics Inc. Kathleen D. Danenberg, BSc, is president and CEO of and holds stock in Response Genetics Inc. The other authors declare no conflicts of interest.

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ISSN: 1556-0864/10/0512-1933

subgroup; the overall response rate to chemotherapy in patients with EGFR-mutant cancers was 47.3%, compared with 23.5% in patients with EGFR wild-type cancers. The clinical implications of this finding have remained unclear. We hypothesized an association between DNA repair capacity and EGFR mutation status as a possible explanation. In this study, we report analysis of a large tumor tissue database for the purported predictive platinum biomarker excision repair cross-complementing 1 (ERCC1), demonstrating that patients with cancers harboring EGFR activating mutations are significantly more likely to exhibit low gene expression levels of ERCC1, a possible explanation for enhanced platinum-based chemotherapy efficacy in this biologically distinct subgroup of NSCLC.

METHODS

Patient Tumor Samples

Formalin-fixed paraffin-embedded tumor samples from patients with NSCLC available from the Response Genetics, Inc. (RGI) database were used for this analysis. In all cases, central pathology review of submitted hematoxylin and eosin-stained slides was conducted by a single pathologist to estimate tumor load per sample and to verify NSCLC histologic subtype as reported by the submitting institution. No further testing was performed to distinguish cell type in cases submitted as not otherwise specified (NOS).

Quantitative Real-Time Polymerase Chain Reaction for ERCC1 mRNA Expression

RNA was isolated from microdissected tumor samples following a proprietary procedure at RGI (Los Angeles, CA; US patent No. 6248,535), a Good Laboratory Practices-compliant and Clinical Laboratory Improvement Amendments-certified commercial laboratory. Briefly, after examination of the hematoxylin and eosin-stained slides, 10-μm thick sections were stained with nuclear fast red to enable visualization of histology for manual or laser capture microdissection. Manual microdissection was performed by excising tumor cells from other tissue with a razor blade and a dissecting microscope on tumor areas greater than 0.5 mm $\times$ 0.5 mm. Laser capture dissection was performed on smaller areas as described previously and ensures that only tumor cells were dissected (P.A.L.M. Microlaser Technologies AG, Munich, Germany).[11] RGI has validated that manual techniques are equivalent to laser technology when tumor areas are greater than 0.5 mm $\times$ 0.5 mm.

The resulting tumor RNA was reverse transcribed into cDNA. Expression of ERCC1 and ACTB (Beta-actin, endogenous reference) was quantified by real-time fluorescence detection of amplified cDNA (ABI PRISM 7900 Sequence Detection System [TaqMan], Perkin-Elmer Applied Biosystems, Foster City, CA). The real-time polymerase chain reaction assay was conducted as described previously.[11,12] All primers were selected using Gene Express software (Applied Biosystems, Foster City, CA) but were adapted to the requirements of cDNA generated from RNA. Previously published sequences of ERCC1 and ACTB were used, and all primers were validated following a previously described protocol.[11,12] All analyses were conducted on all samples in triplicate. The detection of amplified cDNA results in a cycle threshold (Ct) value, which is reciprocal to the amount of cDNA contained in the sample. Normal colon, liver, and St. Universal Mix RNA (Stratagene, La Jolla, CA) were used as control calibrators on each assay plate. Gene expression levels were described as the ratio between two absolute measurements (gene of interest/endogenous reference gene) to control for intersample variation. Before statistical analysis, all ratios were logarithmically transformed including a multiplier, which counted the average cycle threshold values obtained for each gene during the validation process. This procedure facilitated the comparison of samples, which were run on different assay plates.

Scorpion-ARMS for EGFR Mutation Analysis

RGI generated and validated a laboratory developed test using reagents from Qiagen's EGFR29 Mutation test kit, according to manufacturer's instructions. Real-time polymerase chain reaction assays were performed using DNA extracted from formalin-fixed paraffin-embedded samples to detect wild-type or mutant EGFR molecules. By comparing control and mutant sample reactions within our validated range, we were able to detect low levels of mutation of EGFR (1% mutant in a background of wild-type genomic DNA).[13,14] The reagents enabled the detection of the following mutations: 19del, L858R, L861Q, G719X, S768I, and three insertions in exon 20.

Statistical Analyses

The Mann-Whitney U test was used to determine significant associations between continuous variables (i.e., gene expression) and dichotomous variables (i.e., mutation status). Fisher's exact test was used to calculate the significance of associations between two dichotomous variables.

An ERCC1 cut-point value optimized for segregating patient responsiveness to platinum-based therapy in NSCLC was used, as previously described using the maximal χ^2 method.[11] The level of significance was set to $p < 0.05$. All p values reported were based on two-sided tests. All statistical analyses were performed using the Software Packages SPSS for Windows (Version 17.0, Chicago, IL) and JMP 7.0 Software (SAS, Cary, NC).

RESULTS

Patient Characteristics

Specimens from 1207 patients with NSCLC for whom both *ERCC1* gene expression levels and EGFR mutation status were available form the data set for this analysis. By histologic subtype, 712 were adenocarcinomas (ACs), 175 were squamous cell carcinomas, and 320 were NOS or other. Table 1 presents patient characteristics available within the RGI database. Treatment regimens used and therapy-related outcomes were not available for this data set.

Association of EGFR Mutation Status with *ERCC1* Gene Expression

Median ERCC1 mRNA expression was 1.82, with a large intrapatient variability (range, 0.22–27.31) (Table 2).

TABLE 1. Patient Characteristics

Histology	
Adenocarcinoma (AC)	712 (59%)
Squamous cell carcinoma (SCCA)	175 (14.5%)
NOS/other	320 (26.5%)
Age, yr	
Median	67
Range	18–96
Gender	
Male/female	541 (44.8%)/666 (55.2%)
Stage	
Early/late	2 (<1%)/1205 (>99%)

NOS, not otherwise specified.

TABLE 2. *ERCC1* Gene Expression Levels by Histology

	Histology			
ERCC1 mRNA Expression	**NSCLC (All) (N = 1207)**	**AC (N = 712)**	**SCCA (N = 175)**	**NOS/Other (N = 320)**
Median	1.82	1.68[a]	2.42	1.85
Range	0.22–27.31	0.22–11.33	0.51–14.28	0.45–27.31

[a] AC vs. SCCA: $p < 0.001$.

NSCLC, non-small cell lung cancer; AC, adenocarcinoma; SCCA, squamous cell carcinoma; NOS, not otherwise specified; ERCC 1, excision repair cross-complementing 1.

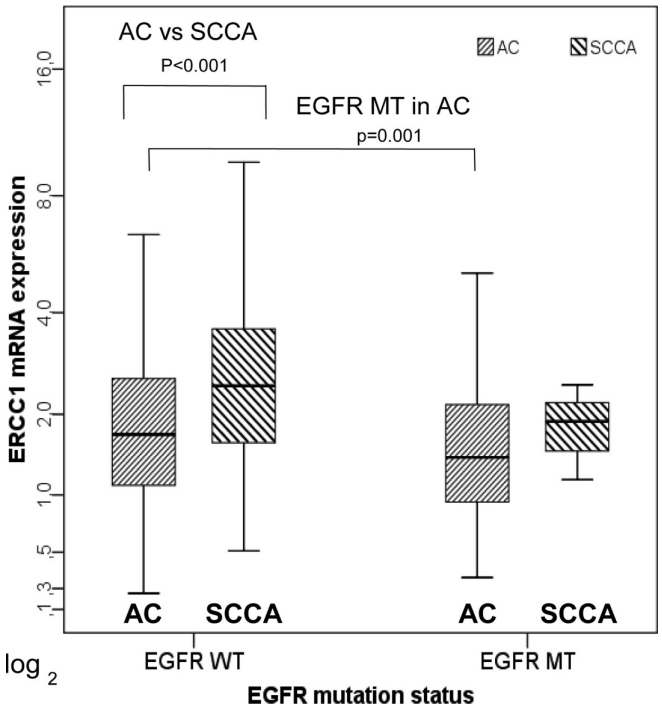

FIGURE 1. Distribution of excision repair cross-complementing 1 (ERCC1) mRNA expression levels by histology and epidermal growth factor receptor (EGFR) mutation status. ERCC1 mRNA expression levels, normalized to the endogenous standard ACTB, were plotted on the *y* axis in log 2. Patients were subdivided into four categories based on EGFR mutation status (wild-type [WT] versus mutant [MT]) and histology: adenocarcinoma (AC) versus squamous cell carcinoma (SCCA). In EGFR-WT tumors, ERCC1 levels were significantly higher in SCCA compared with AC ($p < 0.001$). Significant differences were also observed between AC with WT EGFR versus AC with mutant EGFR ($p = 0.001$), with ERCC1 levels lower in the EGFR-MT subpopulation. The small number of SCCA harboring EGFR mutations precluded statistical analysis.

ERCC1 expression levels were associated with NSCLC histologic subtype.[15,18] For ACs, the median expression level was 1.68 (0.22–11.33), whereas for squamous cancers, the median was 2.42 (0.51–14.28), $p < 0.001$. In the NOS/other category, the median ERCC1 level was intermediate: 1.85 (0.45–27.31). ERCC1 levels were categorized as low (below the reference level of 1.7) in 556 of 1207 patients (46.1%), using a previously described cut point.[15,16] Significantly more ACs were below 1.7 compared with squamous cell cancers ($p < 0.001$) (Figure 1).

Results of EGFR mutation testing showed that 1024 of 1207 specimens were wild type, and 183 were positive for activating mutations (15.2%). Most common were exon 19 deletions (E19del) and L858R missense mutations at 106 of 183 (58%) and 56 of 183 (31%) patients, respectively. Other less common EGFR mutations identified were G719X (presence of G719S, G719A, or G719C), exon 20 insertions (not distinguished), and L861Q. The great majority of EGFR mutations were observed in ACs (144/183, 79%). Within the overall AC population, EGFR-mutant tumors comprised 144 of 712 (20.2%). Only four EGFR activating mutations were found in the 175 patients with squamous cell cancers, 2.3% (three with E19del and one with L858R). In the NOS/other category, EGFR mutations were detected in 35 of 320 (10.9%).

As presented in Table 3, EGFR mutation status was significantly associated with *ERCC1* gene expression levels ($p < 0.001$ by Mann-Whitney U test). In EGFR-mutant versus wild-type cancers, median ERCC1 expression levels were 1.47 (range, 0.32–8.24) and 1.88 (range, 0.22–27.31) respectively. Although ERCC1 expression levels were generally lower in ACs overall, the association of EGFR mutation status and ERCC1 expression was preserved when the AC subset was analyzed separately ($p = 0.001$ in ACs). EGFR-mutant cancers were also more likely to be categorized as ERCC1 low (<1.7) and, therefore, platinum sensitive, $p = 0.002$ by two-sided Fisher's exact test. In the cohort of patients

TABLE 3. Comparison of *ERCC1* Gene Expression with EGFR Mutation Status

ERCC1	EGFR MT	EGFR WT	p
All NSCLC	N = 183	N = 1024	
Median	1.47	1.88	<0.001
Range	0.32–8.24	0.22–27.31	
AC	N = 144	N = 568	
Median	1.42	1.71	0.001
Range	0.32–5.1	0.22–11.33	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; AC, adenocarcinoma; ERCC 1, excision repair cross-complementing 1.

with wild-type EGFR, ERCC1 was significantly lower in AC compared with squamous cell carcinoma ($p < 0.001$).

ERCC1 expression was lower in cancers containing E19del ($n = 106$): median 1.35 (0.32–4.43) versus all other EGFR activating mutations ($n = 77$): ERCC1 median = 1.69, but this difference does not reach statistical significance (0.36–8.24), $p = 0.124$ (Mann-Whitney U test). Similarly, of the two main types of EGFR mutations identified, ERCC1 expression trended toward lower in E19del versus L858R: median = 1.56 (0.36–8.24) ($p = 0.124$, Mann-Whitney U test).

DISCUSSION

Alterations in DNA repair capacity, most commonly related to ERCC1 mRNA or protein expression levels, have been reported to have both prognostic significance and predictive value for platinum-based chemotherapy in patients with NSCLC.[16–21] Although low levels of ERCC1 expression translate into an unfavorable prognosis, conversely, low ERCC1 levels are associated with improved efficacy of platinum-based chemotherapy.[16,19,21] In this study, we report for the first time an association of EGFR mutation status and ERCC1 mRNA expression levels. The great majority of EGFR mutations consist of either in-frame deletions in exon 19 (E19del) or point mutations with substitution of arginine for leucine at amino acid 858 (L858R).[4–6,22] Activating EGFR mutations confer responsiveness to EGFR TKIs through enhanced drug interaction with the adenosine triphosphate-binding site of the tyrosine kinase domain.[4–6] The question of whether patients with NSCLC harboring such tumor mutations are also more responsive to chemotherapy has been raised by the results of the recent IPASS trial and other studies. An association between EGFR activating mutations and ERCC1 expression levels would provide a partial explanation and a mechanism-based rationale for such observations. Nevertheless, a more basic explanation for this association remains unclear.

It has been postulated that impaired nuclear excision repair may be associated with increased genomic instability and increased tumor mutation rates.[19] Nevertheless, EGFR-mutant cancers are generally devoid of the complex genetic abnormalities, which relate to tobacco carcinogenesis.[23] Nevertheless, EGFR and DNA repair pathways have been linked in preclinical studies, possibly through BRCA1, with implications for platinum-based therapy.[24–26] Along this line, cisplatin-induced cytotoxicity has been shown to be dependent on EGFR-directed signaling, resulting in increased platinum-DNA adducts in high EGFR-expressing cell lines.[27] Relatively few data exist on whether EGFR-mutant NSCLC cell lines are more sensitive to DNA-damaging chemotherapy or ionizing radiation in preclinical models.[28–30] Sordella et al.[28] reported that EGFR mutations were associated with activation of antiapoptotic pathways, resulting in decreased sensitivity to a variety of chemotherapeutic agents in vitro, whether DNA damaging or other. On the other hand, Das et al.[29] indicate that EGFR mutation enhances sensitivity to radiation by a multifaceted process, including delayed DNA repair kinetics, suggesting that other components of the DNA repair mechanism may be involved in this association. In accordance with this view, among patients with locally advanced NSCLC treated with radiation and platinum-based chemotherapy, those with EGFR-mutant cancers have recently been reported to have reduced local-regional tumor failure compared with EGFR wild-type cancers (19% versus 46%) and trends toward improved survival: median 62.8 months versus 37.7 months ($p = 0.12$).[31]

The overall incidence of EGFR activating mutations within our data set, 15% (183 of 1207), may seem somewhat high, when compared with that expected within the general U.S. populations of patients with NSCLC. If so, a likely explanation is that the series reported here is partially derived from the RGI testing center and may reflect a patient subgroup more likely to harbor EGFR mutations. As expected, the two most common EGFR mutations identified in this series are E19del and L858R mutations. One intriguing finding is that cancers with E19del tended to have even lower expression levels of ERCC1, when compared with all others or to L858R mutant cancers, although the differences do not reach statistical significance. Patients with cancers harboring E19dels seem to be the most responsive to EGFR TKIs.[32,33] In view of our findings, it is possible that a favorable response profile for E19del cancers extends to platinum-based chemotherapy as well.

In addition to IPASS, some other reports directed toward NSCLC in patients with EGFR mutations also suggest that platinum-based chemotherapy may be advantageous.[34–37] For example, in a report by Hotta et al.[34] in patients with advanced NSCLC receiving first-line chemotherapy, PFS was improved in those with activating EGFR mutations compared with those with wild-type cancers: 6-month PFS of 45.8% versus 21.9% ($p = 0.0422$ in multivariate analysis). Response rate also tended to be higher, 21% versus 15%, although this difference was not significantly different. Further support is provided by a report in Taiwanese patients with EGFR activating mutations receiving first-line chemotherapy, which showed higher response rates with platinum-based therapy versus nonplatinum therapy, 30% versus 9.4%, $p = 0.023$.[36]

Of interest, in a series of surgically resected patients with NSCLC from Korea, Lee et al. have reported that EGFR mutations were more frequent in cancers graded as ERCC1 negative for protein expression by immunohistochemistry. In this series, EGFR mutations were present in 30% of ERCC1-negative cancers versus 12.5% of ERCC1-positive tumors ($p = 0.014$).[38] This report differs from our own in several aspects. First, it assessed ERCC1 by immunohistochemistry-based protein expression rather than mRNA expression levels. Second, the relatively small patient population of 25 patients described in this report consists of early-stage cancers, whereas our population is made up almost entirely of advanced stage patients. Nevertheless, the conclusion that ERCC1 expression is linked to EGFR mutation status is common to both reports.

Several potential alternative explanations exist to explain good patient outcome in patients with NSCLC with EGFR activating mutations, unrelated to our findings. First, not all clinical trials applicable to this question have sug-

gested better than expected chemotherapy efficacy in patients with EGFR-mutant NSCLC. For example, in the First-line Single-agent Iressa vs Gemcitabine and cisplatin trial in Never-smoklers with Adenocarcinoma of the Lung (First-SIGNAL), a Korean trial comparing gefitinib to gemcitabine-cisplatin chemotherapy in a clinical-pathologic selected population similar to IPASS, those patients with EGFR mutations randomized to chemotherapy had a response rate of 37.5%, when compared with 51.9% in EGFR mutation-negative patients.[39] Other studies reporting favorable PFS or overall survival in patients with EGFR-mutated cancers receiving platinum-based chemotherapy may be confounded by the good prognosis portended by EGFR mutation positive status. In the WJTOG3405 study, for example, consisting entirely of EGFR-mutant-positive NSCLC, patients were randomized to gefitinib or docetaxel-cisplatin as first-line therapy. In the chemotherapy arm, the response rate was 32.2% not too dissimilar from that of first-line platinum-based chemotherapy in a general population of advanced stage NSCLC. Nevertheless, disease control rate was favorable at 78%, with a median PFS of 6.3 months and overall survival not yet reached at 15+ months.[9]

As noted earlier, EGFR mutation positivity portends a good prognosis independent of therapeutic intervention, and EGFR mutation-positive cancers are more commonly found in younger patients and females who are never smokers, a group having fewer comorbidities. Finally, EGFR mutations are largely mutually exclusive of KRAS mutations, an additional molecular abnormality portending a poor prognosis.[23]

In summary, we report a significant association between EGFR mutation status and low *ERCC1* gene expression levels in this retrospective analysis of a large tumor tissue database. Therapeutic implications of this association largely remain to be determined, as patient outcomes were not available within our database. An already-initiated molecular profiling trial with a highly annotated patient database regarding therapeutic outcomes, Collaborative Advanced Stage Tissue Lung Cancer (sponsored by the Bonnie J. Addario Lung Cancer Foundation [BJALCF] and it's research arm ALCMI [Addario Lung Cancer Medical Institute]), is equipped to address this association in a prospective manner.

ACKNOWLEDGMENTS

Supported by P30 CA93373, Response Genetics, Inc., UL1 RR024146 from the National Center for Research Resources, American Cancer Society Institutional Research Grant 95-125-07, and the Bonnie J. Addario Lung Cancer Foundation.

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