Tumour gene expression predicts response to cetuximab in patients with KRAS wild-type metastatic colorectal cancer

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BACKGROUND: Although it is accepted that metastatic colorectal cancers (mCRCs) that carry activating mutations in KRAS are unresponsive to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, a significant fraction of KRAS wild-type (wt) mCRCs are also unresponsive to anti-EGFR therapy. Genes encoding EGFR ligands amphiregulin (AREG) and epiregulin (EREG) are promising gene expression-based markers but have not been incorporated into a test to dichotomise KRAS wt mCRC patients with respect to sensitivity to anti-EGFR treatment.

METHODS: We used RT–PCR to test 110 candidate gene expression markers in primary tumours from 144 KRAS wt mCRC patients who received monotherapy with the anti-EGFR antibody cetuximab. Results were correlated with multiple clinical endpoints: disease control, objective response, and progression-free survival (PFS).

RESULTS: Expression of many of the tested candidate genes, including EREG and AREG, strongly associate with all clinical endpoints. Using multivariate analysis with two-layer five-fold cross-validation, we constructed a four-gene predictive classifier. Strikingly, patients below the classifier cutpoint had PFS and disease control rates similar to those of patients with KRAS mutant mCRC.

CONCLUSION: Gene expression appears to identify KRAS wt mCRC patients who receive little benefit from cetuximab. It will be important to test this model in an independent validation study.

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The epidermal growth factor receptor (EGFR) has been implicated in the growth and aggressiveness of a number of different cancers (Mendelsohn and Baselga, 2006), several of which are responsive to drugs that target this receptor (Baselga and Arteaga, 2005; Modjtahedi and Essapen, 2009). The anti-EGFR monoclonal antibody cetuximab has demonstrated clinical benefit in, and is widely used to treat metastatic colorectal cancer (mCRC) (Jonker et al, 2009; Van Cutsem et al, 2010). Efforts have been made to identify biomarkers that will optimise patient selection for treatment to maximise the therapeutic index for mCRC patients receiving cetuximab and other anti-EGFR therapies.

Activating KRAS mutations, which are present in 30–40% of CRC (Samowitz et al, 2000; Andreyev et al, 2001) serve as a key useful biomarker in this context. A number of independent studies strongly link the presence of a somatic activating mutation in the KRAS gene (codons 12 or 13) with nearly complete mCRC resistance to anti-EGFR monoclonal antibodies cetuximab (Benvenuti et al, 2007; Di Fiore et al, 2007; Khambata-Ford et al, 2007; De Roock et al, 2008; Karapetis et al, 2008; Lièvre et al, 2008) and panitumumab (Benvenuti et al, 2007; Amado et al, 2008; Freeman et al, 2008), leading the American Society of Clinical Oncology to recommend that only mCRC patients with KRAS wild-type (wt) mCRC be considered candidates to receive anti-EGFR therapy (Allegra et al, 2009). Other investigated mutations in mCRC are either rare (e.g., activating EGFR mutations (Barber et al, 2004; Tsuchihashi et al, 2005)) or, as a result of conflicting data, controversial with respect to response to anti-EGFR monoclonal antibodies (e.g., PI3K and BRAF); (Samowitz et al, 2005; Di Nicolantonio et al, 2008; Loupakis et al, 2009; Perrone et al, 2009; Prenen et al, 2009; Sartore-Bianchi et al, 2009; Souglakos et al, 2009; Roth et al, 2010). Despite the significance of KRAS mutations, the efficacy of anti-EGFR monoclonal antibodies in the 60–70% of mCRC patients with KRAS wt tumours is still limited, with response rates between 10 and 40% (Allegra et al, 2009). There is a need for additional predictive biomarkers for these patients. Interestingly, the expression of the EGFR protein has not been strongly associated with clinical response to cetuximab in colorectal cancer (Cunningham et al, 2004; Saltz et al, 2004; Chung et al, 2005; Meropol, 2005), although there is limited evidence that amplification of the EGFR gene relates to objective response and other indices of clinical benefit (Moroni et al, 2005; Cappuzzo et al, 2008; Personeni et al, 2008).

Two independent groups recently reported that increased expression of genes encoding two EGFR ligands, amphiregulin (AREG) and epiregulin (EREG) strongly associates with increased therapeutic benefit from cetuximab in mCRC patients (Khambata-Ford et al, 2007; Jacobs et al, 2009; Jonker et al, 2009). Although these results are encouraging, a gene expression-based test with adequate performance has not yet been developed. An expanded model with markers additional to AREG and EREG may be...
optimal. For practical clinical utility the test would categorise patients for treatment, that is, have dichotomising cutpoints (Jacobs et al., 2009).

With the goal of creating a clinically useful test, we have used high-throughput RT–PCR to explore a set of 110 biologically based candidate gene expression biomarkers in formalin-fixed, paraffin-embedded primary tumour specimens from 144 KRAS wt mCRC patients who had been treated with cetuximab mono-therapy. There is a strong rationale for this strategy of testing candidate genes based on the known biology of the EGFR pathway as evidenced by the above referenced association of KRAS to drug resistance and AREG and EREG to drug sensitivity. The fact that constitutive activation of KRAS marks resistance to cetuximab is consistent with the known role of KRAS as a key downstream signal transducer of the EGFR pathway (Mendelsohn and Baselga, 2006). The findings that increased AREG and EREG expression associate with sensitivity to cetuximab accords with the general concept of oncogene addiction (Weinstein, 2002).

MATERIALS AND METHODS

Colorectal cancer tissue samples were obtained from the primary colon tumours removed at initial surgical resection for patients from three cetuximab monotherapy studies: IMC CP02-0144, IMC CP02-0141, and BMS CA225-045. None of the tissue samples were obtained from metastases and a single sample was obtained from the primary tumours. Eligibility in the IMC CP02-0141 study required that patients had previous therapy with at least one chemotherapeutic regimen for advanced disease that included a fluoropyrimidine and irinotecan, and documentation that their disease was refractory to this treatment. Patients were eligible for IMC CP02-0144 and BMS CA225-045 if their disease was refractory to irinotecan-, oxaliplatin-, and fluoropyrimidine-based regimens. In all three studies, eligibility criteria also included an Eastern Cooperative Oncology Group performance status of two or less. In addition, patients were required to be at least 18 years of age; patients in IMC CP02-0144 and BMS CA225-045 could not have received major surgery, radiation chemotherapy, or investigational agents within 4 weeks. Study CP02-0141 also indicated that no surgery was permitted within 21 days of study entry, excluding previous diagnostic biopsy. Standard laboratory test values had to be within normal limits. Patients were treated with cetuximab at a loading dose of 400 mg m–2 followed by 250 mg m–2 weekly. In one study, patients could escalate their dose after the first 3 weeks and every 3 weeks thereafter to a maximum dose of 400 mg m–2, if they did not experience a skin rash greater than grade 2. All protocols were approved by institutional review boards at the participating institutions.

RNA was extracted from a minimum of three to six 5-μm unstained sections of FPE tumour tissue on glass slides without a cover slip, using protocols optimised for colon FPE tissue by Genomic Health Inc. (Redwood City, CA, USA). One slide from each patient was H&E stained and used as a guide for removal of normal-appearing tissue by manual microdissection. RNA was isolated and TaqMan RT–PCR was carried out as previously described (Clark-Langone et al., 2007).

Candidate genes (shown in Supplementary Table S1) were selected for gene expression analysis, based on previous evidence that they may be linked to the sensitivity or resistance to EGFR-inhibitor drugs either directly (as components of the EGFR signaling pathway) or indirectly (by marking fundamental pathobiological variables such as cell cycle activity, or stromal response). As shown in Supplementary Figure S1, using the initial set of 254 samples, expression levels of 99 genes were evaluated. Both emerging evidence regarding the importance of KRAS mutation status in response to EGFR inhibitors and the desire to investigate additional cancer-related genes as well as alternative reference genes resulted in an optimised version of the assay gene panel. Genes significantly associated with outcome in the original assay were carried forward to the optimised assay. Of the original 254 samples, 226 (those with sufficient RNA remaining) were reassayed with the updated panel of 57 genes plus KRAS sequences. All together, 110 genes were evaluated.

A normalisation method was used to compensate for systematic sources of variation in gene expression measurements. In the optimised version of the assay, the cycle threshold (Ct) measurement for each test gene was normalised relative to the average expression of five reference genes (UBB, ATP5E, PGK1, VDAC2, and GPX1) in that tumour RNA sample. The reference genes were selected for this purpose in the course of RT–PCR studies of several hundred genes carried out in over 1800 stage II and III colorectal cancer specimens. For details regarding the assay formats, see Supplementary Figure S1.

Wild-type and mutant KRAS were detected using allele-specific blocker PCR that uses a PCR primer that is specific for the mutant template, and an oligonucleotide that blocks amplification of the wt gene (Morlan et al., 2009). Samples were assayed for the seven most frequent mutations reported in KRAS codons 12 and 13 (Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys, and Gly13Asp), accounting for approximately 97% of all KRAS mutations observed in CRC.

Clinical endpoints and statistical methods

Complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) was assessed by study investigators. Complete response was defined as complete disappearance of all tumour lesions for at least 4 weeks from the date of documentation of CR. Partial response was defined as a decrease (relative to baseline) of 50% or greater in the sum of the products of the two largest perpendicular diameters of all index lesions, as determined by two observations at least 4 weeks apart. Additionally, patients could not meet the criteria for PD. Progressive disease was defined as at least a 25% increase in the sum of the products of all index lesions (taking as reference the smallest sum recorded at or following baseline) or progression of non-measurable lesions or the appearance of any new lesions. Stable disease was defined as failure to meet criteria for CR or PR in the absence of PD. Disease control was defined as having either CR, PR, or SD as a patient’s best response. Objective response rate (ORR) was defined as the proportion of patients achieving a CR or PR as a patient’s best response. Progression-free survival (PFS) was defined as the time from entry onto the study to the first date of progression for patients with documented PD, or the date of death for patients who died within 90 days of their last tumour assessment without documented progression. Patients who did not progress or die were censored at the date of their last tumour assessment. Gene expression was standardised by dividing each gene’s reference-normalised expression level (on the log2 C T scale) by its sample s.d. Univariate logistic regression models were used to examine the associations between the expression of test genes and (clinical) responder and objective response. Univariate Cox proportional hazards regression models were used to examine the associations between the expression of test genes or the four-gene score and PFS. For each regression model, the P-value under the likelihood ratio test of the null hypothesis that the odds ratio (or hazard ratio) equals one is reported. All statistical tests are two-sided and P<0.05 is considered significant. The minimum false discovery rate (FDR) at which each null hypothesis could be rejected using the method of Benjamini and Hochberg (1995) is also provided as a conservative statistical adjustment for the multiplicity of testing multiple gene models.

KRAS expression measurements were used to classify each patient as either positive or negative for at least one KRAS
mutation. Using a logistic regression model, a probability of mutation for each KRAS mutation sequence and sample was estimated. When the predicted probability of a mutation was > 0.5, the sample was classified as positive for the particular KRAS mutation variant. The logistic regression model applied to these data were developed using samples from an external training data set in which the KRAS mutation status of each sample was known. P-values comparing the proportion of responses (disease control or objective response) in patients with and without KRAS mutations were calculated using the χ² test.

Multi-gene classification models were developed using two-layer, five-fold cross-validation approaches in the KRAS wt subset of patients (Ambroise and McLachlan, 2002). A supervised approach was used, analysing only the subset of genes that were significantly associated with outcome, unadjusted P-value < 0.05, * = genes associated with outcome controlling for FDR < 0.05, ** = genes not significantly associated with outcome. In total, 110 genes depicted.

### Table I

| Study      | Disease control endpoint | Objective response endpoint |
|------------|--------------------------|----------------------------|
|            | Non-responder | Responder | Non-responder | Responder | Total |
| CA 225045  | 12 11         | 5 18       | 23            |
| IMCL CP02-0141 | 8 3        | 3 8       | 11            |
| IMCL CP02-0144 | 67 43      | 23 87     | 110           |
| Total      | 87110        | 57110     | 144           |

Abbreviations: CR = complete response; PD = progressive disease; PR = partial response; SD = stable disease. *Includes 1 CR, 30 PR, and 56 SD. **Includes PD only. ***Includes 57 PD and 56 SD.

**Figure 1** (A) Distribution of likelihood ratio P-values in KRAS wt patients by gene rank (disease control). Note: ■ and + = genes significantly associated with outcome, unadjusted P-value < 0.05, ■ = genes associated with outcome controlling for FDR < 0.05, * = genes not significantly associated with outcome. In total, 110 genes depicted. (B) Distribution of likelihood ratio P-values in KRAS wt patients by gene rank (objective response). Note: ■ and + = genes significantly associated with outcome, unadjusted P-value < 0.05, ■ = genes associated with outcome controlling for FDR < 0.05, * = genes not significantly associated with outcome. In total, 110 genes depicted. (C) Distribution of likelihood ratio P-values in KRAS wt patients by gene rank (PFS). Note: ■ and + = genes significantly associated with outcome, unadjusted P-value < 0.05, ■ = genes associated with outcome controlling for FDR < 0.05, * = genes not significantly associated with outcome. In total, 110 genes depicted.
identified as significantly associated with outcome based on univariate analyses of gene expression and outcome (disease control and objective response). Forward stepwise logistic regression was employed in the inner layers and inspection of the area under the curve (AUC) for each of the models was used to identify the optimal multi-gene model. The outer layers were used to evaluate the models constructed in the inner layers. The two-layer, five-fold cross-validation was repeated 50 times using different partitions of the data, yielding 250 models. The threshold for the final four-gene model was determined by identifying the cutpoint that yielded the highest number of true negatives plus true positives on the disease control endpoint. Median time to progression and/or death was calculated using Kaplan–Meier (product-limit estimation) methods and associated 95% confidence intervals were calculated based on the sign test (Brookmeyer and Crowley, 1982). Corresponding Kaplan–Meier plots were used to display PFS for the selected four-gene classifier on the disease control endpoint. The log-rank test was calculated to test the null hypothesis that there is no difference in the probability of progression and/or death over the entire time period among the groups identified by the multi-gene classifier.

RESULTS

Patient characteristics

A total of 326 tumour specimens were obtained from the primary colon tumours removed at initial surgical resection for patients who were enrolled in cetuximab monotherapy trials. After exclusion of 46 specimens based on predefined pathology and laboratory criteria (10 because of insufficient tumour, 22 because of insufficient RNA, and 14 because of laboratory failure), 280 patients had successful gene expression analysis. An additional 26 patients were clinically not evaluable (not treated, or withdrew or died before tumour assessment), resulting in 254 patients that were evaluable for the RT–PCR-based gene expression analysis (174 patients from IMC CP02-0144, 13 patients from IMC CP02-0141, and 39 patients from BMS CA225-045).

An activating KRAS (codons 12 or 13) mutation was identified in 82 (36%) of the 226 patients. Both this frequency, as well as the distribution of the individual codon 12 and 13 mutations (Supplementary Table S2), are in accord with the published literature (Samowitz et al., 2000). Also, consistent with published literature, the disease control, ORR and PFS rates were very low in patients with KRAS mutant compared with KRAS wt tumours (Supplementary Table S3 and Supplementary Figure S2). The 144 patients with KRAS wt tumours were carried forward for gene expression analysis. Of the 144 patients, 1 patient (<1%) had a CR, 30 patients (21%) had a PR, 56 patients (39%) had SD, and 57 (40%) had PD (Table 1).

Gene expression and sensitivity to cetuximab in KRAS wt mCRC

Among the 144 patients with wt KRAS tumours, the expression levels of 26, 12, and 25 genes were associated with the disease control, objective response, and PFS endpoints, respectively (FDR <0.05 after adjusting for multiplicity). As shown by Figures 1A–C, many of these gene expression values had highly significant P-values. Further, as shown in Figure 2, many of these genes are associated with outcome over multiple endpoints (nine of them across all three endpoints). These genes include DUSP6, which encodes a dual specificity phosphatase, and AREG and EREG, which encode the EGFR ligands epiregulin and amphiregulin, respectively. Increased expression of DUSP6, which blocks the EGFR pathway at MAPK, is associated with decreased likelihood of disease control, while increased expression of AREG and EREG, which stimulate the EGFR pathway, are associated with increased likelihood of disease control. Although EREG and AREG tightly co-expressed, they did not co-express with other significant genes, consistent with the possibility that the other genes carry independent predictive information (see gene expression dendrogram in Supplementary Figure S3).

Unlike AREG and EREG, genes encoding other EGFR ligands, TGFalpha, HB-EGF, and EGF, were not consistently associated with outcome. Our results indicate that AREG and EREG tend to be more highly expressed than these other ligands in mCRC (see Supplementary Figure S4).

It should be noted that not all of the highly significant genes are known to be linked to the EGFR pathway. For example, PTP4A3, a gene encoding a protein tyrosine phosphatase that has been reported to be expressed at increased levels in aggressive (Mollevi et al., 2008) or metastatic (Guzinska-Ustymowicz et al., 2009) CRC, strongly associated with therapy benefit across all three endpoints (Figure 2).

Of the three studies combined for this analysis, study IMCL CP02-0144 accounted for 76% of the total patients (Table 1). Results in the subset of patients from study IMCL CP02-0144 alone were highly consistent with results obtained when analysing all three studies combined (data not shown).
Multi-gene classifier developed in patients with KRAS wt mCRC

In view of the substantial fraction of tested genes whose expression was significantly associated with each of the clinical endpoints, we explored the potential performance of multi-gene classifiers. Applying two-layer five-fold cross-validation in patients with KRAS wt tumours, we identified several genes that consistently performed well as a group based on diagnostic measures (AUC, positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity) in the outer layer of the cross-validation. We considered models with differing numbers of genes, including a two-gene model with AREG and EREG only. Evaluation of receiver operating characteristic curves indicated that a two-gene model has a sharper shoulder than a four-gene model, which implies that this model is more sensitive to the choice of a cutpoint than the four-gene model. The additional robustness of the four-gene model to choice of cutpoint, combined with the desire to minimise analytical variation suggested that a four-gene model was optimal. A final model that yields a predictive score includes four genes: EREG, AREG, and two other genes, DUSP6 (which encodes a dual-specificity phosphatase) and SLC26A3 (which encodes an intestinal chloride ion transporter that is also known as downregulated in colon adenoma).

A cutpoint in the four-gene score was selected to construct a classifier that identifies KRAS wt patients most likely to benefit from cetuximab treatment. The cutpoint for the four-gene model is the maximum of the sum of the true negative and true positive values for the disease control endpoint when the predictive score was applied. As shown in Figure 3A, this selected cutpoint would lead to treatment of 84 patients (58% of KRAS wt patients) out of which 71 would have disease control (85% of these treated patients). Sixty patients would not have been treated, 27% of whom would have disease control (the majority (82%) of whom had SD).

When the four-gene score with the same cutpoint is applied to objective response, 84 patients would be treated, and 28 (33%) would respond; 60 patients would not be treated, and 3 of these (5%) would respond (Figure 3B). With respect to both the disease control and objective response endpoints, use of this multi-gene classifier in addition to KRAS status significantly improves specificity and PPV when compared with a patient selection based on KRAS status alone, but with a slight decrease in sensitivity (Figure 3C and D).

The performance of the four-gene classifier with respect to PFS is shown in Figure 4. The median PFS for KRAS wt patients with a four-gene score at or above the cutpoint is 163 days compared with a median PFS for all KRAS wt patients of 103 days. It is noteworthy that the 60 KRAS wt patients with a four-gene score below the cutpoint had a median time to progression and/or death similar to the 82 patients whose tumours carried a KRAS mutation (40 and 41 days, respectively; hazard ratio for this comparison is 1.04 (95% CI: 0.74–1.46)). The hazard ratio for comparison of KRAS wt patients above and below the four-gene score cutpoint is 0.22 (95% CI: 0.15–0.32). A test of the proportional hazards assumption indicated that the assumption was not violated (P = 0.41). However, it should be stated that the performance of the four-gene classifier on the PFS endpoint is evaluated here in the same group of patients that was used to develop the classifier based on objective response and disease control endpoints.

DISCUSSION

Here, we report that a number of candidate gene expression markers, many of which were selected based on their relationship to the EGFR pathway, strongly associate with disease control, PFS and ORR in KRAS wt mCRC patients receiving monotherapy with the anti-EGFR antibody cetuximab. Multivariate analysis
evaluating gene expression associated with disease control yields a four-gene model that predicts the likelihood that individual patients with KRAS wt mCRC will respond to cetuximab. This model sharply dichotomises patients with respect to likelihood of response, which has practical potential in the clinical setting. We propose that this model is sufficiently promising that it should be brought forward for testing in a clinical validation study.

Two of the genes in this model, AREG and EREG, have been previously identified as strong markers of sensitivity to anti-EGFR antibodies: by one study that analysed metastatic mCRC tissue from patients receiving cetuximab monotherapy (Khambata-Ford et al, 2007), and by another study that analysed primary mCRC tissue from patients receiving cetuximab in combination with chemotherapy (Jacobs et al, 2009; Jonker et al, 2009). Although AREG and EREG have not been studied in a large validation trial with a non-treated patient arm, our results with those of the two previous studies provide substantial evidence that these two markers are real and robust.

To develop an optimised gene expression-based test, we have examined additional candidate gene expression markers. The discoveries that key regulatory genes in the EGFR pathway, in the form of KRAS, EREG, and AREG, are anti-EGFR predictive markers suggested to us the potential value of screening other candidate markers based on both the biology of this pathway as well as other features of the pathobiology of CRC. As this study examines primary mCRC tissue from patients receiving cetuximab monotherapy, it is unencumbered by the potentially confounding complication presented by combination therapy. Further, it incorporates both a relatively large number of KRAS wt patients and multiple clinical endpoints.

The strength of association of many of these markers with clinical endpoints is remarkable: nine genes associating with all three endpoints (FDR < 0.05) after adjusting for multiple comparisons. Although further work needs to be done to confirm the performance of the identified markers, a number of them are compatible with known biology, in particular, the concept of oncogene addiction (Weinstein, 2002). Thus, AREG and EREG, which encode activators of the EGFR pathway, associate with increased likelihood of clinical benefit; DUSP6, which encodes a phosphatase inhibitor of the EGFR pathway, associates with decreased likelihood of clinical benefit.

Although this study does not include a control arm of untreated patients, and therefore leaves open the possibility that a number of the described biomarkers are prognostic rather than predictive of cetuximab benefit, it seems probable that many of the top genes identified are in fact predictive of drug response because the objective response endpoint lends itself to discovery of predictive rather than prognostic biomarkers. An objective response in colon cancer is generally recognised as a treatment effect.

We performed multivariate analysis on the entire set of identified markers to generate a four-gene classifier that, in exploratory analysis, performs better than EREG/AREG. Used in conjunction with KRAS mutation status, this assay associates with several indices of clinical outcome better than either gene expression alone or KRAS mutation status alone.

The four-gene classifier is exploratory, as this model was discovered and estimated using the same KRAS wt patients. The degree of over-fitting was assessed in part through two-layer, five-fold cross-validation. External validation of this multi-gene model will require an independent study that is adequately statistically powered and includes a control arm of patients who are not treated with cetuximab.

Our findings are based on analysis of formalin-fixed primary tumour tissue, which is generally obtained at the time of definitive colorectal tumour resection. Therefore, if this classifier were to be validated, it will be technically feasible to introduce it into clinical practice. The applicability of these results to patients treated with cetuximab in combination therapy deserves further study.

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Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)
