Genomic markers of panitumumab resistance including ERBB2/HER2 in a phase II study of KRAS wild-type (wt) metastatic colorectal cancer (mCRC)

Garrett S. Barry¹, Maggie C. Cheang², Hector Li Chang¹, Hagen F. Kennecke³

¹Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of British Columbia, Wesbrook Mall, Vancouver, BC V6T 2B5, Canada
²The Institute of Cancer Research, Sutton, London, Surrey SM2 5NG, United Kingdom
³Medical Oncology, BC Cancer Agency, Vancouver, BC V5Z 4E6, Canada

Keywords: mCRC, nanostring, ERBB2, HER2, EGFR inhibitor resistance

ABSTRACT

A prospective study was conducted to identify biomarkers associated with resistance to panitumumab monotherapy in patients with metastatic colorectal cancer (mCRC). Patients with previously treated, codon 12/13 KRAS wt, mCRC were prospectively administered panitumumab 6mg/kg IV q2weeks. Of 34 panitumumab-treated patients, 11 (32%) had progressive disease at 8 weeks and were classified as non-responders.

A Nanostring nCounter-based assay identified a 5-gene expression signature (ERBB2, MLPH, IRX3, MYRF, and KLK6) associated with panitumumab resistance ($P = 0.001$). Immunohistochemistry and in situ hybridization determined that the HER2 (ERBB2) protein was overexpressed in 4/11 non-responding and 0/21 responding cases ($P = 0.035$). Two non-responding tumors had ERBB2 gene amplification only, and one demonstrated both ERBB2 amplification and mutation. A non-codon 12/13 KRAS mutation occurred in one panitumumab-resistant patient and was mutually exclusive with ERBB2/HER2 abnormalities.

This study identifies a 5-gene signature associated with non-response to single agent panitumumab, including a subgroup of non-responders with evidence of aberrant ERBB2/HER2 signaling. KRAS wt tumors resistant to EGFRi may be identified by gene signature analysis, and the HER2 pathway plays an important role in resistance to therapy.

INTRODUCTION

Colorectal cancer (CRC) has high rates of metastasis and recurrence [1, 2]. Therapy for advanced disease includes epidermal growth factor receptor (EGFR)-inhibitors, including cetuximab and panitumumab, which have only shown effectiveness in RAS non-mutated tumors, though demonstrating a marked range of clinical response rates [3–5]. This heterogeneous response rate may be due to substantial intrinsic molecular differences among these tumors, including, but not limited to, mitogen-activated protein-kinase (MAPK/ERK) and phosphoinositide 3-kinase (PI3K/Akt) pathways, and anti-apoptosis [6–8]. Phase III trials of patients with metastatic KRAS unselected mCRC treated with single agent EGFR inhibitors (EGFRi) reported a high rate of resistance to therapy, with 70% of patients having progressive disease as best response [3]. Excluding patients with KRAS mutated tumors and other recently described RAS mutants [9, 10], reduces this proportion to approximately 30% of patients without response to EGFRi therapy. Determining which patients are unlikely to respond to EGFRi therapy will spare the patient from treatment-related toxicity and allow them to be directed to other therapeutic options or clinical trials, thus gaining time.

Inter-tumoral heterogeneity in CRC has been demonstrated in whole genome expression array studies, with the identification of six and three biological subtypes in two large, recent cohorts, respectively [7, 11]. Furthermore, gene expression-based drug sensitivity predictors have been shown to be superior to other genomic, epigenomic, and proteomic drug sensitivity predictors across human breast cancer cell lines [12]. Gene
expression signatures already have an established role in risk stratification and treatment selection in some settings, most notably in early stage breast and colon cancers [13–15].

In this phase II biomarker study, we aimed to identify molecular markers in KRAS wt mCRC that would identify patients resistant to EGFRi therapy. We determined the presence of molecular determinants of primary resistance to EGFRi therapy, defined as disease progression within 8 weeks of commencing single agent therapy with panitumumab. Gene expression profiling was conducted with a knowledge-based selection of candidate genes and targeted next-generation sequencing was performed. ERBB2/HER2 copy number status and protein expression quantification was conducted due to the identification of this gene in the response signature.

RESULTS

Of the 37 evaluable patients, one demonstrated a false negative KRAS codon 12 mutation and an additional two patients did not have sufficient primary tumor nucleic acid isolation; these patients were removed from subsequent analyses (Figure 1). Of 34 remaining evaluable cases, the median age was 64.5 years. All patients received prior chemotherapy including 59% who received 5-Fluorouracil, irinotecan and oxaliplatin and 53% received prior bevacizumab. Response to therapy and median number cycles of panitumumab are summarized in Table 1. Eleven of the remaining 34 patients developed progressive disease (PD) (median # of cycles = 4) at the time of the initial imaging for response at the 8-week time point and were classified as non-responders. Of the remaining 23 patients, nine had stable disease (SD) and eight had a partial response (PR) (median # cycles = 12). Six patients demonstrated a best response of two-year prolonged stable disease (median # cycles = 22). For the purposes of the biomarker analysis, patients were dichotomized into response groups and defined as non-responders if they had PD (n = 11) and responders if they had SD, PR, or PSD.

Gene expression analysis identified 5 genes significantly associated with non-response to panitumumab

We identified five highly ranked genes with a positive correlation with panitumumab resistance below an FDR of 15% by Significance Analysis of Microarrays (SAM); ERBB2, MLPH, IRX3, MYRF, and KLK6 (Figure 2). Two of these genes, ERBB2 and MLPH, had a FDR of 0%. These two genes alone were borderline significantly associated with non-response to panitumumab (P = 0.05, CI 95% = 0.0015–1.98), but the five-gene signature demonstrated a more significant association (P = 0.001, CI 95% = 0.597–2.16). Recognizing the weakness of a small cohort size, we performed normalization with the 220 cases of KRAS wt mCRC from the TCGA cohort. Ninety-five of the 120 genes had matched expression between BCCA and TCGA cohorts with over 30% of cases above background expression after normalization. Performing identical subsequent analyses on the TCGA-normalized data identified four highly ranked genes with an FDR below 10%, ERBB2, MLPH, IRX3, and MYRF (Supplementary Figure 2), and demonstrated very significant association with panitumumab response (P = 2.86 × 10⁻⁵, CI 95% = 1.02–1.89). A comparison of all three analyses can be found in Supplementary Figure 3. While re-performing the analysis on a smaller set of genes may have inflated the level of significance, taken together, this analysis bolstered the significant genes found in the SAM analysis prior to this normalization method. We noted that EGFR expression was not significantly associated with panitumumab response in any of these analyses, despite being the direct target of panitumumab.

Mutations in RAS, BRAF, and ERBB2

Thirty-one out of 34 evaluable primary tumor cases included in the study had sufficient genomic DNA and matching blood DNA to be sequenced by the Illumina-based OncoPanel. Ten out of 11 matching metastatic tumor samples also passed these criteria and were sequenced.

Of the 31 primary tumor samples, one KRAS mutation was found in a non-responding patient (Figure 3). This G to A transition mutation produced an A146T amino acid change. A mutation in BRAF (K601E) was also found in this KRAS A146T mutant patient. This A146T mutation was also identified in a second patient with PD that was ultimately excluded from the study on the basis of unavailable RNA for gene expression analysis. One NRAS mutation at Q61L was also identified in a patient with progressive disease. No HRAS mutations were identified.

A total of four BRAF mutations were identified in the 31 patients, two in patients with PD and two in patients with SD. One of the two patients with PD had the V600E mutation and the other with a K601E mutation. Of patients with SD, one had a K601E mutation and the other had an L597R mutation. One patient classified as having a partial response had a frame shift mutation in EGFR itself, likely abrogating EGFR pathway activation.

There was one patient with a mutation in ERBB2 classified as a non-responder. This was a homozygous V747L mutation that occurred in the tyrosine kinase domain of the protein; however, we are unaware of any study reporting the functional significance of this mutation at the time of this study.

Three patients had PDGFRA mutations, of which two were EGFRi non-responders. One of these PDGFRA mutations occurred in a resistant patient with a coexisting NRAS mutation. KIT mutations were found in two patients within the responder group. Infrequent mutations in MTOR and PIK3CA were also found in both responders and non-responders.
Immunohistochemistry and FISH demonstrate over-activity of the HER2 pathway in a subset of RAS-BRAF wt panitumumab non-responders

Of the 35 assessable TMA cases, two did not undergo the gene expression assay and one had KRAS mutated codon 12, so were excluded as described earlier. Twenty-six out of 32 cases were negative (score = 0), 5/32 cases were low (score = 1), and 4/32 cases were high (score = 3+) for HER2 protein expression (Figure 3). The level of HER2 protein expression was significantly associated with resistance to panitumumab (Fisher’s exact test, \( P = 0.035 \)).

FISH was also performed on cases of the TMA with adequate tumor tissue and KRAS wt as for IHC, removing the same 3 cases from a total of 37. An additional 9 cases did not have adequate FISH results for assessment, leaving 25 cases with measurable ERBB2 copy number. ERBB2 amplification was correlated significantly with panitumumab resistance (\( P = 0.009 \), CI 95% 0.418–2.68). Four cases were found to have amplification of ERBB2 with HER2/CEP ratios of > 2.1 (Figure 3). Three of these amplified cases demonstrated PD after panitumumab and had average copy numbers exceeding 8 copies of ERBB2. The fourth amplified case had HER2/CEP ratio of 2.2 and demonstrated stable disease.

**DISCUSSION**

The objective of this prospective, exploratory biomarker study was to identify biomarkers associated with resistance to panitumumab therapy in KRAS wt mCRC on archival paraffin embedded tissue. Eleven out of 34 patients (32%) with KRAS wt (codons 12/13) mCRC did not respond to single agent panitumumab. A 5-gene signature was developed including ERBB2 and MLPH on the Nanostring® nCounter platform, and strongly associated with non-response to therapy. HER2 protein amplification was present in 13.8% (4/29) of all-RAS wt cases and was significantly associated with non-response to therapy.

A recently described 180-gene predictor assay was found to significantly classify EGFR-inhibitor-treated lung
and colon adenocarcinoma disease types into high and low response groups [16]. The authors found 26 genes from the 180-gene set were adequate to significantly classify high and low cetuximab responders in \texttt{KRAS}\textsuperscript{wt} mCRC. These genes were represented in the PI3K/AKT and MAPK pathways downstream of EGFR. A separate phase I dose-escalation cetuximab trial in a \texttt{KRAS}\textsuperscript{wt} mCRC cohort reported a non-overlapping set of 6 genes associated with response to EGFR inhibition, though the biological significance of these genes is currently unknown [13]. None of these 6 genes were represented in the expression signature of the current study.

A subset of poor-prognosis \texttt{BRAF}-mutant-like (\texttt{BRAFm}) patients, defined as having tumors with gene expression profiles similar to known \texttt{BRAFm} tumors, were previously identified in a \texttt{KRAS}\textsuperscript{wt} CRC cohort using a predictor based on differential expression of 32 gene pairs [17]. Remarkably, the \texttt{BRAFm} predictor identified 13% of \texttt{KRAS}\textsuperscript{wt} CRC patients with a much poorer prognosis ($P = 0.022$), capturing a significant number of non-\texttt{BRAF} mutant patients with a \texttt{BRAFm} expression signature. We included these 64 genes in the present study, reasoning a \texttt{BRAF}-mutant-like phenotype may be associated with non-response to EGFR-directed therapies. Indeed, \texttt{MYRF}, \texttt{MLPH}, and \texttt{IRX3} expression - genes implicated in the \texttt{BRAF}-mutant-like phenotype - were associated with non-responders. Overexpression of these three genes was most significantly associated with panitumumab non-response aside from \texttt{ERBB2}, and thus may represent a relevant molecular pattern of panitumumab resistance by aberration and/or bypass of the EGFR signaling pathway for cancer cell survival. \texttt{IRX3} may be implicated in colorectal tumorigenesis by reducing tumor cell sensitivity to the Dpp/TGF\beta pathway, granting tumor cells a growth advantage [18]. \texttt{MLPH} and \texttt{MYRF} have not been specifically described as linked to EGFRi resistance in CRC tumors, so this is a novel finding of this study.

The presently described outcomes are comparable with other studies. Peeters \textit{et al}. reported 16% of patients with \texttt{KRAS} wt (codons 12/13/61) tumours showed no objective response to panitumumab monotherapy [9]. Among patients with \texttt{KRAS} wt (codons 12/13/61), nine and 13 out of 138 patients showed mutations in \texttt{NRAS} and \texttt{BRAF}, respectively, all of which showed no objective response to panitumumab. Patients with mutant \texttt{TP53} and \texttt{PIK3CA} showed response rates of 17 and 20%, respectively. Doulliard \textit{et al}. found that additional mutations in \texttt{KRAS}, \texttt{NRAS}, and \texttt{BRAF} in patients with \texttt{KRAS} wt codons 12/13 conferred an inferior progression free survival and overall survival for those treated with panitumumab-FOLFOX4 therapy [10]. Seventeen percent of \texttt{KRAS} wt codons 12/13 patients had these additional \texttt{RAS} mutations. Of note, one patient in our study had an A146T mutation in \texttt{KRAS}, unreported by routine clinical

### Table 1: Characteristics and response to therapy of 34 patients with previously treated \texttt{KRAS}\textsuperscript{wt} mCRC

|                          | Total | 34 (%) |
|--------------------------|-------|--------|
| **Median age**           |       | 64.5   |
| **Prior Therapy**        |       |        |
| 5-Fluorouracil (5-FU)/Capecitabine (C) only | 10 (29) |
| 5-FU/C and Irinotecan    | 1 (3)  |
| 5-FU/C and Oxaliplatin   | 3 (9)  |
| 5-FU/C, Irinotecan and Oxaliplatin | 20 (59) |
| **Prior Bevacizumab**    |       |        |
| Yes                      | 18 (53) |
| No                       | 16 (47) |
| **Response to therapy**  |       |        |
| Progressive Disease      | 11 (32) |
| Stable Disease           | 9 (26)  |
| Prolonged Stable Disease | 6 (18)  |
| Partial Response         | 8 (24)  |
| Complete Response        | 0 (0)   |
| **Median number of cycles of q2weekly therapy** |       |        |
| Progressive Disease      | 4      |
| Stable Disease           | 8      |
| Partial Response/Prolonged Stable Disease | 16 |
hotspot mutation assays. Previous evidence suggests that \textit{KRAS} A146T mutations may have oncogenic potential in other cancer types [18–20]. Of note, the \textit{KRAS} mutant patient also bore a K601E mutation in \textit{BRAF}, an exceedingly rare occurrence. We suspect that the two mutations may have contributed to a lack of response to panitumumab in this patient [21].

Interestingly, four \textit{BRAF} mutations were identified in the cohort, two in patients with resistant tumors and two in patients with stable disease. \textit{BRAF} mutation is a powerful prognostic marker in mCRC conferring a significantly shorter overall survival regardless of type of systemic therapy [22, 23]. Despite some evidence [22], mutations in \textit{BRAF} have not been consistently established as a predictive marker for EGFRi resistance, and current treatment recommendations do not exclude these patients from EGFRi therapy. However, in a large randomized clinical trial of irinotecan plus panitumumab versus irinotecan alone, the latter was favored in cases harboring \textit{BRAF} mutations [24]. The variability of \textit{BRAF} as a marker of EGFRi resistance is reflected in this study as \textit{BRAF} mutations occurred in both 20% of resistant (PD) and 9.5% of responsive (SD/PR/PSD) tumors. This may highlight the value of a gene response signature to further distinguish resistant from responsive disease in \textit{BRAF} mutant mCRC.

The significance of mutations found in \textit{PDGFR\alpha} and \textit{KIT} are currently unclear. Activating \textit{PDGFR} mutations have been implicated in a favorable tumor microenvironment and angiogenesis [25]. While possible that a relationship exists between \textit{PDGFR} mutation and lack of response to EGFRi therapy, the low frequency of these mutations in the present cohort, as well as a confounding \textit{NRAS} mutation, was not adequate to support this. However, the CORRECT clinical trial of multi-kinase (including \textit{PDGFR}) inhibitor regorafenib in mCRC patients who previously failed panitumumab and/or cetuximab demonstrated a significant benefit both in progression-free and overall survival (HR = 0.5 and 0.71, respectively) [26]. This seems to suggest that a variety of tyrosine kinases, including \textit{PDGFR}, may be involved in tumor resistance to agents targeting specific oncogenic targets, such as EGFRi. There have been no reports of \textit{KIT} involvement in EGFRi responsiveness in the colorectal

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{Gene expression results. (A) Unsupervised hierarchical clustering of 34 cases of the \textit{KRAS} wt mCRC cohort (BCCA) by 120 selected genes. Metastatic cases substituted primary tumor cases when available. (B) SAM analysis of selected 120 genes ranked two top genes with 0% FDR, \textit{ERBB2} and \textit{MLPH}. Cases were ordered by weighted average score of these two genes from low to high. Response to panitumumab was added and shows clustering with higher weighted average score ($P = 0.05$, CI 95\% = 0.0015–1.98). (C) With an FDR cutoff of 15\%, the addition of three genes, \textit{IRX3}, \textit{MYRF}, and \textit{KLK6}, improved clustering of weighted average score with panitumumab non-responders ($P = 0.001$, CI 95\% = 0.597–2.16). Non-responders represented patients with progressive disease (black), while responders encompassed stable disease, partial response, and prolonged stable disease (white).}
\end{figure}
cancer literature, and the presence of these mutations in the responder group is of unknown significance.

We queried the TCGA data for alterations in the 5 top ranked genes from our gene expression analysis using the cBioPortal for Cancer Genomics from Memorial Sloan Kettering Cancer Center [27, 28]. Thirteen (6%) of the CRC cases from the TCGA database (N = 224) had gene alterations in ERBB2, 7 of which were gene amplifications (3.1%). All ERBB2 amplified cases were mutually exclusive of alterations in MYRF, MLPH, IRX3, and KLK6. KLK6 was altered in 4 cases (2%), MYRF was altered in 3 cases (1%), and IRX3 was altered in 2 cases (1%). MLPH was altered in 1 case. The relatively low frequency of these aberrations may point to their specificity with regards to predictors for EGFRi resistance.

Of the genes we included in the expression analysis, ERBB2 overexpression by FISH and IHC was strikingly and significantly associated with panitumumab non-response. Previous studies have reported a rate of HER2 positivity of 2.0 to 4.3% as defined by HER2 protein expression of 3+ protein or FISH amplification > 2.1 [29–32] in colorectal tumors. One study reported an overall frequency of 2.3% in 2349 patients reported in the literature [33]. Concordance of HER2 positivity in paired samples was reported to be 96% [32].

Until recently it was thought that HER2 gene copy number was not predictive of EGFR-directed therapy response [34]. However, recent studies do suggest HER2 overexpression by gene amplification may indeed be related to poor outcome in KRAS wt mCRC patients treated with cetuximab or panitumumab [35]. In a study of 137 patient-derived xenograft (PDX) tumors, HER2 amplification was documented in a 13.6% of cases in patients with cetuximab resistant, KRAS wild-type tumors [33], a frequency similar to the one documented in the current study. Two representative KRAS wt, HER2 amplified, cetuximab resistant PDX tumors were treated with pertuzumab, an anti-HER2 monoclonal antibody preventing dimerization with HER partners including EGFR, in combination with either lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor, or cetuximab, and resulted in substantial and prolonged tumor regression, whereas pertuzumab alone had no effect. An earlier phase II study of herceptin, an anti-HER2 monoclonal antibody, plus cytotoxic therapy with irinotecan in patients with advanced HER2 positive (2+ or 3+ protein expression) mCRC, reported a response to therapy in 5 of 7 patients, but the study was closed due to poor accrual as only 8% of 138 screened patients were HER2 positive [29]. In the current study 4 of 29 (13.8%) patients with all-RAS wt tumors had
HER2 overexpression by IHC and/or FISH. This incidence significantly increases the feasibility of a trial of a dual EGFR inhibitor combined with HER2 directed therapy in advanced HER2 positive mCRC. Indeed, a recent phase II study of combination trastuzumab plus lapatinib in KRAS wt and HER2 IHC 2/3+ and FISH + cancers met its primary efficacy endpoint and reported a 32% response rate in patients with advanced, treatment refractory cancer [36]. This lends strong support to ERBB2 as relevant therapeutic biomarker in mCRC.

In conclusion, this study represents a primary effort to define a novel gene expression signature based on differentially expressed genes in KRAS wt mCRC not responsive to EGFRi, in particular, panitumumab monotherapy. A five-gene expression signature was found to be highly associated with panitumumab resistance in mCRC, with ERBB2 and MLPH as the top differentially expressed genes. We intend to explore if this gene expression signature is unique to mCRC or has utility in other tumor sites as well. The secondary goal was to identify possible mechanisms of panitumumab resistance by targeted resequencing of implicated genes of the EGFR and HER2 signaling pathways and protein expression and gene amplification of HER2 and ERBB2, respectively. We detected molecular aberrations associated with EGFRi resistance in 6 out of 10 of non-responding patients. Further modeling and validation of the gene expression signature in a larger, independent cohort may allow refined prediction of resistant cases. A prospective screening method to identify patients least likely to respond to panitumumab monotherapy will spare patients from unnecessary exposure to EGFRi administration and toxicity and will help to redirect these patients to an alternative therapy or clinical trial earlier, enhancing their chances of a positive outcome.

MATERIALS AND METHODS

Patient eligibility and study design

A total of 40 patients with KRAS wt (codons 12 and 13) advanced mCRCs with measurable disease were prospectively enrolled at three BCCA cancer centers on an exploratory biomarker study. Patients were previously treated with 5-Fluorouracil and received, or were ineligible for, prior oxaliplatin/5-FU and irinotecan and were treated with panitumumab 6mg/kg IV q2w until progression or toxicity. None of the patients enrolling on this study had ever been exposed to prior anti-EGFR therapy, either as a single agent or in combination with an irinotecan-based regimen. Computed Tomography (CT) was done at baseline.

Table 2: List of gene mutations in 31 primary tumors by best overall response to panitumumab treatment identified within the Illumina-based sequencing oncopanel

| Gene  | Progressive Disease (PD) | Stable Disease (SD) | Partial Response (PR)/Prolonged stable disease (PSD) |
|-------|--------------------------|---------------------|---------------------------------------------------|
|       | N = 10 (%)               | N = 8 (%)           | N = 13 (%)                                        |
| BRAF  | 2 (20)                   | 1 (13)              | 0                                                 |
| EGFR  | 0                        | 0                   | 1 (8)                                             |
| KRAS  | 2 (20)                   | 0                   | 0                                                 |
| HRAS  | 0                        | 0                   | 0                                                 |
| NRAS  | 1 (10)                   | 0                   | 0                                                 |
| AKT1  | 0                        | 0                   | 0                                                 |
| ALK   | 0                        | 0                   | 0                                                 |
| ERBB2 | 1 (10)                   | 0                   | 0                                                 |
| IDH1  | 0                        | 0                   | 0                                                 |
| IDH2  | 0                        | 0                   | 0                                                 |
| KIT   | 0                        | 0                   | 2 (15)                                            |
| MAPK1 | 0                        | 0                   | 0                                                 |
| MAP2K1| 0                        | 0                   | 0                                                 |
| MTOR  | 0                        | 3 (38)              | 4 (31)                                            |
| PDGFR 2| 2 (20)                   | 0                   | 1 (8)                                             |
| PIK3CA| 0                        | 1 (13)              | 1 (8)                                             |
| PTEN  | 0                        | 0                   | 0                                                 |
| STAT1 | 1 (10)                   | 1 (13)              | 1 (8)                                             |
| STAT3 | 1 (10)                   | 0                   | 2 (15)                                            |
| TP53  | 9 (90)                   | 7 (88)              | 11 (85)                                           |
and every 8 weeks to assess response. The primary endpoint was tumor response and patients were classified by standard RECIST criteria as Progressive Disease (PD), Stable Disease (SD), Prolonged Stable Disease ≥ 24 weeks (PSD) or Partial Response (PR). For the biomarker analysis, patients with PD were classified as non-responders and patients with SD, PSD24 or PR were classified as responders to panitumumab. The primary objective of the study was to determine rates of response and non-response by RECIST to single agent panitumumab therapy and the study was powered to detect biomarkers with a frequency of 20–50%. The study was approved by the institutional REB and was registered on clinicaltrials.gov study NCT00853931.

Sample collection, experimental steps, and analytical workflow are summarized in Figure 1. Of 40 KRAS wt mCRC patients treated with panitumumab monotherapy, 37 were evaluable for response to therapy. One patient with a KRAS mutation identified in codon 12 that was not identified by standard clinical testing was excluded from all analyses. Two samples that did not have sufficient RNA for the Nanostring assay requirements, nor did not pass expression data quality control determined by nSolver software (Nanostring), were also excluded, leaving a total of 34 patients included in the final analysis.

Gene expression profiles

Gene selection and probe design

A 120-gene Nanostring nCounter codest set was designed from previously reported gene sets predictive of BRAF mutation status and cetuximab response in mCRC [13, 17, 37], and a hypoxia signature predictive of metastasis in a broad range of tumor types [38]. Additional genes of interest such as RAD51, EGFR, ERBB2, and CRYAB were also included [35, 39–42]. The complete gene list can be found in Supplementary Table 1.

Nucleic acid isolation and expression profiling

Total ribonucleic acid (RNA) and genomic deoxyribonucleic acid (gDNA) was isolated from duplicate 1mm diameter cores taken from FFPE tissue blocks with a Total Nucleic Acid Extraction kit (Qiagen). Qubit and Nanodrop-1000 spectrophotometry measured quantity and quality of RNA, respectively. Samples with a minimum quantity threshold of 12.5 ng/ul and a 260/280 quality ratio between 1.7 and 2.3 were included in the nCounter assay as previously described [43]. The nCounter assay was performed following recommended protocols using between 100 and 250 ng of total RNA.

Data analysis

Gene expression data analysis

Data processing was performed in R Statistical Environment. The BCCA study cohort gene expression data was normalized to the same dimensional space as the wt cases from the 220-case TCGA cohort and sample-to-sample geomean normalized using eight housekeeping genes: ACTB, GUSB, PSMC4, RPLP0, PUM1, SF3A1, TFRC, and MRPL19. Genes with expression above background in fewer than 30% of cases after normalization were removed. Principal component analysis (PCA) was used to visualize the dimensional space of the BCCA and TCGA cohort datasets.

Two-class unpaired Significance Analysis of Microarrays (SAM) was used to identify the top ranked genes associated with resistance to panitumumab in the BCCA cohort. For the cases where matched primary- and metastatic-derived tumor samples were both available, metastatic samples were included for SAM analysis in place of primary tumor samples. False Discovery Rate (FDR), or q-value, was used to identify the genes most significantly associated with panitumumab response. A T-statistic, called the score(d), was calculated by calculating a “d” statistic for each gene based on its linear regression with the outcome. “d” statistics are ordered and plotted against their expected order statistics after a large number of random permutations of the response data. The score(d) represented how far the “d” statistic is from the expected order statistic and was used to weight each corresponding gene within a signature equation. Weighted average expression of top ranked genes was calculated in each analyses and association with panitumumab response was performed by two-tailed unpaired Welch’s T-test, which reports a 95% confidence interval for the difference in means of responders vs. non-responders.

Since the BCCA study cohort was composed entirely of KRAS wt tumors, we sought to identify if there was any expression bias of 120 selected gene expression profiles within a larger mixed KRAS wt and mutant CRC tumor cohort from The Cancer Genome Atlas (TCGA) database. Two hundred and twenty colon adenocarcinoma tumor samples with both global gene expression profiles and somatic mutational analysis were collected from TCGA. Gene expression and somatic mutation data from the TCGA cohort were previously measured with a custom Agilent 244K array and both SOLiD 4 and Illumina HiSeq platforms, respectively. Global gene expression profiles were abridged to only represent the 120 candidate genes.

Since the BCCA cohort size was relatively small and represented a biased population based in terms of KRAS wt status, we determined whether a gene expression bias was present between known KRAS mutant and wt cases. We assessed this by principal component analysis (Supplementary Figure 1). By normalizing the BCCA cohort dataset to the much larger TCGA cohort, the BCCA cohort dataset shifted to overlap with the TCGA cohort dataset correcting platform variation between datasets.

DNA sequencing

Targeted resequencing of a panel of common oncogenes and tumor suppressor genes using an Illumina-based
Oncopanel was performed. This sequencing panel included genes suspected to confer resistance to EGFR-directed therapy beyond KRAS codons 12 and 13 mutations, such as in other regions of the RAS homologs. A minimum of 250 ng of genomic DNA was submitted for Illumina-based Oncopanel targeted resequencing. The coding exonic sequence and at least 2bp of flanking intronic sequence of each target was determined from the source DNA material following RainDance-mediated primer-DNA droplet merging, PCR amplification and Illumina sequencing. Resulting sequences were aligned to the GRCh37 human genome reference using BWA (version 0.7.5a, mem algorithm). Variant calling was performed using VarScan2 (v2.3.6), with the minimum allele frequency threshold for reporting variants set to 0.1. cDNA nucleotide numbering begins at the A of the initiating codon (ATG) as per HGVS convention of the reference sequences (Supplementary Table 2).

Immunohistochemistry and fluorescence in situ hybridization

As ERBB2 gene expression showed the highest association in tumours that did not respond to panitumumab, immunohistochemistry (IHC) of HER2 protein and ERBB2 in situ hybridization (ISH) analysis was conducted. The antibody used for IHC was specific to HER2 protein (Ventana Medical Systems, Inc.). HER2-stained tissue microarray (TMA) slides were scored as 0 for negative, 1–2 for moderate expression, and 3+ as positive overexpression based on stain intensity according to the ASCO/CAP guidelines. Scores were defined as negative (0–2) or positive (3+) and analyzed by Fisher’s exact test for significance.

For FISH analysis, TMA slides were stained with the Inform HER2 Dual ISH DNA Probe Cocktail Assay (Ventana Medical Systems, Tucson AZ) to determine HER2 gene amplification. Adequate staining was defined as the presence of both signals in at least 20 nuclei, performed on duplicate cores when possible. The numbers of HER2 and chromosome 17 signals were enumerated in 20 nuclei (only in nuclei containing both signals). HER2:Chromosome 17 ratios were calculated by dividing the number of HER2 signals by the number of chromosome 17 signals in each case; ratios of ≥ 2 were considered to have Her2 amplification. Significance of ISH results as a continuous variable to response was analyzed by Welch's two-tailed T-test, also reporting a 95% confidence interval for the difference in means between responders vs. non-responders.

Adjacent signals were only considered separate if the distance between the signals was greater or equal to one signal. Small clusters of HER2 signals where individual signal counting was impossible were counted as 6 signals, and large clusters were counted as 12 signals, as per the manufacturer’s specifications.

Abbreviations

BRAF<sup>m</sup>: BRAF-mutant-like; CT: Computed Tomography; CRC colorectal cancer; EGFR: epidermal growth factor receptor; EGFRi: EGFR inhibitors; FDR: False Discovery Rate; FISH: fluorescence in situ hybridization: gDNA: genomic deoxyribonucleic acid; IHC: immunohistochemistry; ISH: in situ hybridization; MAPK/ERK: mitogen-activated protein–kinase; mCRC: metastatic colorectal cancer; PCA: Principal component analysis; PD: Progressive Disease; PI3K/Akt: phosphoinositide 3-kinase; PR: Partial Response; PSD: Prolonged Stable Disease; SAM: Significance Analysis of Microarrays; SD: Stable Disease; TCGA: The Cancer Genome Atlas; TMA: tissue microarray; wt: wild-type

ACKNOWLEDGMENTS

We would like to thank Aly Karsan of the BC Cancer Research Centre, Rod Docking of the Genome Sciences Centre, Samuel Leung and Dongxia Gao of the Genetic Pathology Evaluation Centre, the Centre for Translational and Applied Genomics staff at the BC Cancer Agency, and Julie Lorette at the BC Cancer Agency Histology Laboratory.

FUNDING

This study was funded in part by AMGEN CANADA, the BC Cancer Foundation and the BC Cancer Agency. Maggie Chon U Cheang is supported by the Cancer Research UK (CRUK) Core grant (Grant number C1491/A15955) to Clinical Trials and Statistics Unit at The Institute of Cancer Research (ICR-CTSU).

CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to declare.

This study was funded in part by Amgen Canada. Amgen Canada was not involved in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. No conflicts arise from this funding as the five gene signature would preclude patients from receiving Panitumumab (made by Amgen).

A patent has been filed for some of the five gene signature described in the manuscript.

REFERENCES

1. Jemal A, Simard EP, Dorell C, Noone A-M, Markowitz LE, Kohler B, Eheman C, Saraiya M, Bandi P, Saslow D, Cronin KA, Watson M, Schiffman M, et al. Annual Report to the Nation on the Status of Cancer, 1975–2009, Featuring
the Burden and Trends in Human Papillomavirus (HPV)–Associated Cancers and HPV Vaccination Coverage Levels. Journal of the National Cancer Institute. 2013; 105: 175–201. doi: 10.1093/jnci/djs491.

2. Canadian Cancer Society’s Advisory Committee on Cancer Statistics, Canadian Cancer Statistics 2014. Canadian Cancer Society. 2014.

3. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. Journal of clinical oncology. 2008; 26:1626–1634.

4. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomasic G, Penna C, Ducreux M, Rougier P, Renault-Llorca F, Laurent-Puig P. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res. 2006; 66:3992–3995.

5. Heinemann V, Stintzing S, Kirchner T, Boeck S, Jung A. Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. Cancer Treat Rev. 2009; 35:262–271.

6. Calistri D, Rengucci C, Seymour I, Lattuneddu A, Polifermo AM, Monti F, Saragoni L, Amadori D. Mutation analysis of p53, K-ras, BRAF genes in colorectal cancer progression. J Cell Physiol. 2005; 204:484–488.

7. Roepman P, Schlicker A, Tabernero J, Majewski I, Tian S, Roepman P, Schlicker A, Tabernero J, Majewski I, Tian S. Mutations in vertebrate pancreas: A comprehensive survey in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in the RAS-MAPK, PI(3)K (phosphatidylinosito...
22. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, Agarwal A, Maru DM, Sieber O, Desai J. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer. 2011; 117:4623–4632.

23. Eklof V, Wikberg ML, Edin S, Dahlin AM, Jonsson BA, Oberg A, Rutegard J, Palmqvist R. The prognostic role of KRAS, BRAF, PIK3CA and PTEN in colorectal cancer. Br J Cancer. 2013; 108:2153–2163.

24. Seymour MT, Brown SR, Middleton G, Maughan T, Hsu H, Earle M, Brufsky A, Evans T, Richman S, Gwyther S, Lowe C, Seligmann JF, Wadsley J, Maisey N, Chau I, Hill M, Dawson L, et al. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. The Lancet Oncology. 2013; 14:749–759.

25. Estevez-Garcia P, Castano A, Martin AC, Lopez-Rios F, Garcia-Carbonero R, PDGFRalpha/beta and VEGFR2 polymorphisms in colorectal cancer: incidence and implications in clinical outcome. BMC Cancer. 2012; 12:514.

26. Grothey A, Van Cutsem E, Sobrero A, Sassi F, Sinicrope FA, Safran H, Wong MK, Earle M, Brufsky A, Evans T, Richman S, Gwyther S, Lowe C, Seligmann JF, Wadsley J, Maisey N, Chau I, Hill M, Dawson L, et al. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. The Lancet Oncology. 2013; 14:749–759.

27. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012; 3:300–342.

28. Network TCGA. (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature, pp. 330–337.

29. Ramanathan RK, Hwang JJ, Zamboni WC, Sinicrope FA, Safran H, Wong MK, Earle M, Brufsky A, Evans T, Troetschel M, Walko C, Day R, Chen HX, et al. Low overexpression of HER-2/neu protein expression in colorectal cancer limits the usefulness of trastuzumab (Herceptin) and eribulin as therapy. A phase II trial. Cancer Invest. 2004; 22:858–865.

30. Schuell B, Gruenberger T, Scheithauer W, Zielinski C, Wriba F. HER 2/neu protein expression in colorectal cancer. BMC Cancer. 2006; 6:123.

31. Kavanagh DO, Chambers G, O’Grady L, Barry KM, Waldron RP, Bennani F, Eustace PW, Tobbia I. Is overexpression of HER-2 a predictor of prognosis in colorectal cancer? BMC Cancer. 2009; 9:1471–2407.

32. Sclafani F, Roy A, Cunningham D, Wotherspoon A, Peckitt C, Gonzalez de Castro D, Tabernero J, Glimelius B, Cervantes A, Eltahir Z, Oates J, Chau I. HER2 in high-risk rectal cancer patients treated in EXPERT-C, a randomized phase II trial of neoadjuvant capcitabine and oxaliplatin (CAPOX) and chemoradiotherapy (CRT) with or without cetuximab. Ann Oncol. 2013; 24:3123–3128.

33. Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, Cora D, Di Nicolantonio F, Buscarino M, Petti C, Ribero D, Russolillo N, Muratore A, et al. A molecularly annotated platform of patient-derived xenografts (“xenopatients”) identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. Cancer Discov. 2011; 1:508–523.

34. Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, van Cleeft PH, van Krieken JH, Punt CJ, Nagtegaal ID. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. Eur J Cancer. 2010; 46:1997–2009.

35. Martin V, Landi L, Molinari F, Fountzilas G, Geva R, Riva A, Saletti P, De Dossos S, Spitala A, Tejpars S, Kalogeras KT, Mazzucchelli L, Frattini M, et al. HER2 gene copy number status may influence clinical efficacy to anti-EGFR monoclonal antibodies in metastatic colorectal cancer patients. Br J Cancer. 2013; 108:668–675.

36. Siena S, Bartore-Bianchi A, Trusolino L, Martino C, Bencardino K, Lonardi S, Leone F, Zagonel V, Bertotti A, Valtorta E, Siravegna G, Amatu A, Vanzulli A, et al. Therapeutic dual inhibition of HER2 pathway for metastatic colorectal cancer (mCRC): The HERACLES trial. 2015 Gastrointestinal Cancers Symposium: J Clin Oncol 33, 2015 (suppl 3; abstr 565).

37. Balko JM, Black EP. A gene expression predictor of response to EGFR-targeted therapy stratifies progression-free survival to cetuximab in KRAS wild-type metastatic colorectal cancer. BMC Cancer. 2009; 9:1471–2407.

38. Hu Z, Fan C, Livasy C, He X, Oh DS, Endw MG, Carey LA, Subramanian S, West R, Ikpat F, Olopade OI, van de Rijn M, Perou CM. A compact VEGF signature associated with distant metastases and poor outcomes. BMC Med. 2009; 7:1741–7015.

39. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Erkan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, Shimizu T, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med. 2011; 3:3002442.

40. Tsang JY, Lai MW, Wong KH, Chan SK, Lam CC, Tsang AK, Yu AM, Tan PH, Tse GM. alphaB-crystallin is a useful marker for triple negative and basal breast cancers. Sci Transl Med. 2011; 3:3002442.

41. Eltahir Z, Oates J, Chau I. HER2 in high-risk rectal cancer patients treated in EXPERT-C, a randomized phase II trial of neoadjuvant capcitabine and oxaliplatin (CAPOX) and chemoradiotherapy (CRT) with or without cetuximab. Ann Oncol. 2013; 24:3123–3128.
42. Tennstedt P, Fresow R, Simon R, Marx A, Terracciano L, Petersen C, Sauter G, Dikomey E, Borgmann K. RAD51 overexpression is a negative prognostic marker for colorectal adenocarcinoma. Int J Cancer. 2013; 132:2118–2126.

43. Nielsen T, Wallden B, Schaper C, Ferree S, Liu S, Gao D, Barry G, Dowidar N, Maysuria M, Storhoff J. Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using formalin-fixed paraffin-embedded breast tumor specimens. BMC Cancer. 2014; 14:1471–2407.