Tumor budding predicts response to anti-EGFR therapies in metastatic colorectal cancer patients

Inti Zlobec, Francesca Molinari, Vittoria Martin, Luca Mazzucchelli, Piercarlo Saletti, Rosangela Trezzi, Sara De Dosso, Tatjana Vlajnic, Milo Frattini, Alessandro Lugli

Results: Tumor buds and K-RAS mutation both correctly classified 68% of patients. All patients with K-RAS mutation \((n=7)\) or high-grade tumor budding \((n=11)\) were non-responsive, of which 4 patients had both features. All 13 partial responders were K-RAS wild-type with low-grade tumor budding. Combined, the predictive value of K-RAS and tumor budding was 80%. Additionally, high-grade tumor budding was significantly related to worse progression-free survival \([HR (95\% CI): 2.8 (1.3-6.0, P = 0.008)]\).

Conclusion: If confirmed in larger cohorts, the addition of tumor budding to K-RAS analysis may represent an effective approach for individualized patient management in the metastatic setting.

© 2010 Baishideng. All rights reserved.

Key words: Anti-epidermal growth factor receptor therapy; Colorectal cancer; K-RAS; Prognosis; Tumor budding
bined systemic chemotherapies. Monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) such as cetuximab and panitumumab have recently been approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%[2,3]. Several molecular and protein biomarkers are currently being intensively investigated for their potential predictive value including K-RAS, B-RAF, PIK3CA and PTEN. Although recent randomized clinical trials have not been unanimous concerning the predictive value of K-RAS on outcome, the vast majority of studies to date do support a lack of responsiveness in patients with mutation[4-9]. These data have led the American Society of Clinical Oncology, Food and Drug Administration and European Medicines Agency to recommend that patients with mCRC be tested for K-RAS gene mutation before administration of EGFR-targeted therapies[10]. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

In colorectal cancer, tumor buds, defined as dedifferentiated single cells or clusters of up to 5 cells at the invasive tumor front, are considered the histological hallmark of epithelial mesenchymal transition and are thought to be responsible for the subsequent steps in invasion and metastasis[11,12]. Although tumor buds can be observed using regular hematoxylin and cosin (HE) slides, evaluation is facilitated by pan-cytokeratin stains[12]. Tumor budding has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is highly predictive of both lymph node and distant metastasis stage[13-25]. Moreover, most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer (AJCC/UICC)[26]. In addition, we have previously shown that tumor budding is not related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in mCRC patients treated with anti-EGFR-based therapies[27]. Therefore, the aim of this study was to evaluate the predictive and prognostic value of tumor budding and determine its complementary value to K-RAS gene status in mCRC patients treated with cetuximab or panitumumab-based regimens.

MATERIALS AND METHODS

Patients and specimen characteristics

Forty-three consecutive patients with histologically confirmed mCRC treated at the Oncology Institute of Southern Switzerland, Bellinzona, Switzerland with cetuximab or panitumumab-based regimens were entered into this retrospective study. Cetuximab was administered at a standard loading dose of 400 mg/m² over 2 h, followed by a weekly dose of 250 mg/m² over 1 h. Single agent panitumumab 6 mg/kg every 2 wk was administered to 2 patients who were refractory to oxaliplatin- and irinotecan-based regimens. With the exception of 2 patients who received cetuximab as frontline therapy, the others had failed at least one prior chemotherapy regimen. For those who progressed on irinotecan-based regimens, cetuximab was administered in combination with these regimens given at the same dose and schedule. Therefore, patients were selected based on evidence that treatment outcome was attributable only to the administration of cetuximab or panitumumab. Treatment was continued until progressive disease (PD) or toxicity occurred. Response was assessed every 6-8 wk by means of computerized tomodensitometry or nuclear magnetic resonance. The Response Evaluation Criteria in Solid Tumors were adopted for evaluation and objective tumor response was classified into complete response (CR), partial response (PR), stable disease (SD) and PD[28]. Accordingly, only patients who achieved either CR or PR were considered as responders.

Assay methods

K-RAS, B-RAF and PIK3CA mutational status: Formalin-fixed paraffin-embedded surgical resection specimens were available for all patients. We searched for point mutations in K-RAS exon 2 (including codons 12 and 13), BR-RAF exon 15 (including codon 600) and PIK3CA exons 9 and 20 (including codons 542, 545 and 1047). All samples were subjected to automated sequencing by ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA) and analysed with appropriate software (Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

Epidermal growth factor receptor: fluorescent in situ hybridization

The EGFR gene status evaluation was performed by fluorescent in situ hybridization (FISH) on 3-μm thick tissue sections. Tissue sections were treated using Paraffin Pretreatment Kit II (Abbott Molecular AG, Baar, Switzerland) according to the manufacturers instructions. Dual-colour FISH assay was performed using LSI EGFR/CEP7 probes (Vysis). The LSI EGFR probe is labelled in SpectrumOrange and covers an approximately 300 kb region that contains the entire EGFR gene at 7p12. The CEP7 probe, labelled in SpectrumGreen, hybridises to the α satellite DNA located at the centromere of chromosome 7 (7p11.1-9.1). Target sections and probe were co-denatured at 75°C for 5 min and allowed to hybridise overnight at 37°C. Post-hybridisation stringency wash was carried out in a water bath at 72°C for 5 min. After washing twice and drying at room temperature for 10 min, slides were mounted with 46-diamidino-2-phenylindole (DAPI II, Abbott Molecular). Fluorescent in situ hybridization signals were evaluated with a Zeiss Axioscobe equipped with single and triple band pass filters. Images for documentation were captured using an AxioCam camera and processed using the AxioVision system. Patients
showing two of chromosome 7 in the vast majority of cells were classified as eusomic. Patients with an aberrant number of chromosome 7, defined as more than 4 in at least 50% of cells, were classified as markedly polysomic. Patients with a ratio more than 3 between the EGFR gene and chromosome 7 centromere signals in at least 10% of cells were classified as having EGFR gene amplification.

**Immunohistochemistry**

Immunohistochemistry staining was performed for both CK22 (an epithelial cell marker facilitating the visualization of tumor buds) and PTEN. Paraffin-embedded tissue blocks were cut at 3 μm. Whole tissue sections were de-waxed and re-hydrated in dH2O. Following pressure cooker-mediated antigen retrieval in 0.001 mol/L ethylenediaminetetraacetic acid pH 8.0, endogenous peroxidase activity was blocked using 0.5% H2O2. Sections were incubated with 10% normal goat serum for 20 min. After incubation with primary antibody (PTEN Ab-4, NeoMarkers, Fremont, CA, USA; 1:50 and CK22 polyclonal, Genetex, Inc, 1:100), sections were incubated with HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature, immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min, and counterstained with haematoxylin. PTEN protein expression was detected mainly at the cytoplasmic level, although occasional nuclear positivity was present. PTEN negative tumors were those showing a dramatic reduction or absence of immunostaining in at least 50% of cells, as compared with the internal control. The evaluations were performed without knowledge of clinical data or the results of other analyses.

**Assessment of tumor budding**

Tumor budding was defined as dedifferentiated single cells or clusters of <5 cells at the invasive tumor front. In all cases, the tumor invasive front was scanned at low power using a 5 × objective lens and the region of densest tumor budding was identified. The number of tumor buds within this region was counted using a 40 × objective lens. Evaluation was performed blinded to clinical endpoints. Inter-observer agreement was assessed between independent observers (Lugli A, Vlajnic T, Zlobec I). Discordant cases were discussed until agreement was reached. High-grade tumor budding was defined as 15 tumor buds/HPF.

**Study design**

The study was designed as a retrospective analysis. The main objective was to correlate response to anti-EGFR-based therapies with pathological and molecular findings. The secondary endpoint was represented by the correlation of tumor budding with progression-free survival (PFS) and overall survival.

**Statistical analysis**

Threshold values for determining high-grade vs low-grade tumor budding were assessed using receiver operating characteristic curve analysis with 100- bootstrapped replications of the data. The sensitivity, specificity, positive predictive value and negative predictive value (NPV) for high-grade tumor budding, EGFR amplification or copy number gain, K-RAS, B-RAF, PIK3CA and loss of PTEN as well as their association with response were evaluated by simple logistic regression analysis. Inter-observer variability of tumor budding (low-grade/high-grade) was assessed by the κ statistic and by investigating the percentage of concordance between independent observers. Univariate and multivariable PFS time differences stratified by tumor budding and after adjustment for K-RAS mutational status were evaluated using simple and multiple Cox regression analysis, respectively, after verification of the proportional hazards assumption. The Kaplan-Meier method was used to illustrate PFS time differences by tumor budding grade. Fisher’s Exact test was used to determine the association of tumor budding for response in subgroup analysis. Finally, classification and regression tree analysis (CART) methods were used to determine the features best predicting response to treatment. The CART trees were fitted using DTREG statistical software. To assess the amount of overfitting, 100 10-fold cross-validation experiments were performed. In each of those 100 experiments, the data set was randomly split into 10 smaller data sets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. To resolve uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher’s exact test to test for a relationship between tumor budding, K-RAS mutation and response to therapy. Given the significant association of both these features with response, CART analysis was performed for patients with low-grade tumor budding and K-RAS wild-type gene status only. A second CART analysis was performed conditioning only on K-RAS wild-type patients.

**RESULTS**

**Patient characteristics**

The present study analyzed forty-three patients, 26 men (60%) and 17 women (40%). Patient characteristics and response by treatment with anti-EGFR monoclonal antibodies are summarized in Table 1. Median survival time was 37.3 mo (range 3.6-180) and PFS time was 16.0 mo (range 1-171). Thirteen patients (30%) achieved PR after cetuximab- or panitumumab-based therapy.

**Association of tumor budding with molecular features**

The percentage of concordance between observers was 88% with a κ value of 0.6. Tumor budding and K-RAS gene status was evaluable in all cases. High-grade tumor budding occurred in 11 cases (25.6%) while low-grade tumor budding was found in the remaining 32 patients (74.4%). Tumor budding was not significantly associated with either EGFR status (P = 0.95), K-RAS (P = 0.43),...
Zlobec I et al. Tumor budding and anti-EGFR therapies

Table 1 Characteristics of metastatic colorectal cancer patients treated with anti-epidermal growth factor receptor therapy (n = 43) n (%)  

| Clinico-pathological feature | Frequency |
|-----------------------------|-----------|
| Age (yr), median (range)    | 64 (26-82) |
| Gender                      |           |
| Male                        | 26 (60.5)  |
| Female                      | 17 (39.5)  |
| Response                    |           |
| Progressive disease         | 19 (44.2)  |
| Partial response            | 13 (30.2)  |
| Stable disease              | 11 (25.6)  |
| EGFR                        |           |
| No Amplification/gene copy number gain | 4 (10.5) |
| Amplification/gene copy number gain | 34 (89.5) |
| K-RAS                       |           |
| Wild-type                   | 32 (74.4)  |
| Mutation                    | 11 (25.6)  |
| B-RAF                       |           |
| Wild-type                   | 38 (88.4)  |
| Mutation                    | 5 (11.6)   |
| PIK3CA                      |           |
| Wild-type                   | 41 (95.4)  |
| Mutation                    | 2 (4.7)    |
| PTEN                        |           |
| Negative                    | 12 (27.9)  |
| Positive                    | 31 (72.1)  |
| Overall survival time (mo), median (range) | 37.3 (3.6-180) |
| Progression-free survival (mo), median (range) | 16.0 (1-171) |
| Number of tumor buds, median (range) | 9.0 (1-44) |
| Tumor budding               |           |
| High                        | 11 (25.6)  |
| Low                         | 32 (74.4)  |

EGFR: Epidermal growth factor receptor.

B-RAF (P = 0.598), PIK3CA (P = 0.451) or PTEN expression (P = 0.241) (data not shown).

Association of tumor budding with response

The predictive ability of each feature for response is shown in Table 2. High-grade tumor budding was significantly associated with no objective response (P = 0.011). In fact, all patients with PR had low-grade tumor budding (sensitivity 100%) and all patients with high-grade tumor budding were PD or SD (negative predictive value, NPV: 100%). The overall accuracy of tumor budding for response was 68.3%.

K-RAS gene status was evaluated in all 43 patients and 11 (25.6%) were identified as mutated while the remaining 32 cases (74.4%) were wild-type. A significant association of K-RAS mutation with no objective response was observed (P = 0.011). Moreover, all patients achieving PR were K-RAS wild-type (sensitivity 100%) while K-RAS mutated cases were all non-responders (NPV 100%). As for tumor budding, the overall accuracy of K-RAS for response was 68.3%.

Of the 19 patients with wild-type K-RAS and no response, high-grade tumor budding was able to identify an additional 7 non-responder patients (Table 3). Together, the combined overall accuracy of tumor budding and K-RAS increased from 68.3% to 80% with a sensitivity of 100% for PR and an improvement in specificity to 72.1% with only 12/43 cases in this series misclassified with these two parameters alone.

Algorithm for patient classification using tumor budding, EGFR, K-RAS, B-RAF, PIK3CA and PTEN

Since the predictive accuracy for response using tumor budding combined with K-RAS mutation was 80%, the classification of wild-type K-RAS/low-grade tumor budding patients was further investigated using the remaining molecular parameters and analyzed by CART (Figure 1A). For the remaining 25 patients, negative expression of PTEN occurred in 6 cases and 5/6 (83%) were not responders. Of the remaining 16 patients with positive PTEN expression and available EGFR status, all cases with amplification or copy number gain (n = 13, three were not evaluable for EGFR gene status) had a PR. PIK3CA and B-RAF gene status did not contribute predictive information in this setting which included tumor budding. Moreover, of the 43 patients, 4 cases were misclassified, leading to 90.7% of patients being classified into appropriate response groups.

In order to compare the performance of this algorithm conditioned on K-RAS and tumor budding to an algorithm conditioned only on K-RAS, we performed a second CART analysis to classify patients with wild-type K-RAS gene status into response groups using the remaining molecular features, as described above (Figure 1B). Using this approach, PTEN expression, followed by B-RAF mutation and EGFR amplification or copy number gain were included in the analysis. PIK3CA was not a predictive factor here, most likely due to the low frequency (n = 2) of patients with mutation in this cohort. Seven of the 43 patients were incorrectly classified leading to an overall classification rate of 83.7%.

An overview of the predictive accuracies of K-RAS, tumor budding, K-RAS plus tumor budding, as well as the two algorithms including and excluding tumor budding is presented in Figure 2. In particular, the accuracy of either tumor budding alone or K-RAS analysis alone was 68.3%. This value improved to 80% when analyzing the combined accuracy of budding with K-RAS gene status. The predictive ability of a 4-panel combination of features including K-RAS/PTEN/B-RAF/EGFR was 83.7%. Among the features evaluated, the combined analysis of K-RAS/tumor budding/PTEN/EGFR demonstrated an overall accuracy of 90.7% for response to anti-EGFR agents.

Tumor budding, K-RAS and PFS

When evaluating PFS, high-grade tumor budding was significantly linked to an increased relative risk [HR (95% CI): 2.8 (1.3-6.0), P = 0.008] (Figure 3). In addition, when evaluating both tumor budding and K-RAS mutation status in multivariable analysis, high-grade tumor budding maintained its negative effect on clinical outcome [HR (95% CI): 2.78 (1.3-6.0), P = 0.022],
Table 2  Predictive ability of each feature for partial response

| Feature                      | Total No. of patients | No. of correctly predicted PR | No. of correctly predicted PD + SD | No. of PD + SD predicted as PR | No. of PR predicted as PD + SD | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Accuracy (%) | P-value |
|------------------------------|-----------------------|------------------------------|-----------------------------------|-------------------------------|--------------------------------|----------------|----------------|---------|---------|-------------|---------|
| Budding (< 15 cells)        | 43                    | 13                           | 11                                | 19                            | 0                              | 100            | 40.6          | 37      | 100     | 68.3        | 0.011   |
| EGFR (no AMP/CNG)           | 38                    | 10                           | 4                                 | 24                            | 0                              | 100            | 29.4          | 14      | 100     | 57.1        | 0.556   |
| K-RAS (wild-type)           | 43                    | 13                           | 11                                | 19                            | 0                              | 100            | 40.6          | 37      | 100     | 68.3        | 0.011   |
| B-RAF (wild-type)           | 43                    | 13                           | 5                                 | 25                            | 0                              | 100            | 34.2          | 17      | 100     | 58.3        | 0.301   |
| PIK3CA (wild-type)          | 43                    | 13                           | 2                                 | 28                            | 0                              | 100            | 31.7          | 7       | 100     | 53.3        | 1.0     |
| PTEN (positive)             | 43                    | 12                           | 11                                | 19                            | 1                              | 91.7           | 38.7          | 37      | 92.3    | 64.5        | 0.07    |

PD: Progressive disease; SD: Stable disease; PR: Partial response; EGFR: Epidermal growth factor receptor; PPV: Positive predictive value; NPV: Negative predictive value; AMP/CNG: Amplification or copy number gain.

Figure 1  Algorithm illustrates the classification of patients into response groups. Non-response: Patients with progressive disease or stable disease. A: Classification and regression tree (CART) analysis was performed for patients with K-RAS wild-type/low tumor budding. CART identified a significant contribution of PTEN and epidermal growth factor receptor (EGFR) to the classification of responsive and non-responsive patients. Thirty-nine patients correctly classified (90.7%); B: CART analysis performed for patients with K-RAS wild-type tumors identifying a significant contribution of PTEN, B-RAF and EGFR to the classification of responder and non-responder patients. Thirty-six patients correctly classified (83.7%). AMP/CNG: Amplification or copy number gain; mCRC: Metastatic colorectal cancer.

while K-RAS was not linked to PFS [HR (95% CI): 1.54 (0.8-3.1), P = 0.236].

**DISCUSSION**

The aim of this study was to determine whether tumor budding is a predictive or prognostic factor in mCRC patients treated with anti-EGFR-based therapies. Our results show that high-grade tumor budding predicts non-response in these patients and in combination with K-RAS mutation may correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable PFS also in a K-RAS-independent manner.

We found no association between high-grade tumor budding and K-RAS gene mutation in this series of mCRC patients. Using two entirely independent cohorts of 88 and 117 patients, respectively, we have previously shown this lack of association between K-RAS and tumor budding, although mutation in codon 12 and 13 was observed in 38.6% of all high-grade tumor budsers. Our findings here using a third independent cohort are in agreement with these results. In contrast, Prall and Oswald documented in 95 sporadic CRC patients, a significant association between mutation and tumor budding, and moreover, independently of invasion growth patterns. Our differing results may be explained by the types of tumor specimens used (paraffin-embedded vs fresh frozen), differences in molecular analysis (DNA sequencing vs PCR-RFLP) and notably by the choice of methods of evaluation (tumor buds only vs tumor buds plus cytoplasmic pseudo-fragments).

We document here a significant association between high-grade tumor budding and a lack of objective response in patients with mCRC treated with anti-EGFR therapies. Tumor budding has been significantly related to unfavourable clinical and histopathological features including higher tumor grade, vascular invasion, lymph node...
Table 3 Tumor budding and K-RAS followed by PTEN, epidermal growth factor receptor, B-RAF and PIK3CA stratified by response group

| Response | K-RAS | Tumor budding | PTEN (AMP/CNG) | EGFR | B-RAF | PIK3CA |
|----------|-------|---------------|----------------|------|-------|--------|
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT    | WT     |
| NR       | WT    | Low           | NEG            | Yes  | WT   |

Cases which were correctly predicted using K-RAS and tumor budding. EGFR: Epidermal growth factor receptor; AMP/CNG: Amplification or copy number gain; MUT: Mutation; NE: Not evaluable; NEG: Negative; NR: Non response; POS: Positive; PR: Partial response; WT: Wild-type.

metastasis, distant metastasis, local recurrence, and poorer overall and disease-specific survival time independently of TNM stage. Additionally, tumor budding is inversely related to dense peritumoral lymphocytic inflammation at the invasive front suggesting that the pro-budding phenotype may be tempered by specific immune responses. We have recently reported that a high ratio of CD8+ tumor buds in non-metastatic CRC was found to be a more important prognostic factor than either CD8+ T-lymphocytes or tumor budding alone. Although we evaluated CD8+ cells in these 43 specimens and their ratio with tumor budding, we did not find any predictive or prognostic value of CD8+ in this series, suggesting that the immune response may not play a role in conferring response in these treated, metastatic patients (data not shown). On the other hand, high-grade tumor budding was not only associated with non-response to anti-EGFR therapies but all patients with this unfavourable feature were non-responsive. In addition, we found a significantly shorter PFS in patients with high-grade tumor budding independently of K-RAS, supporting the predictive and prognostic effect of this histomorphological feature among this cohort of patients.

An association between K-RAS gene mutation and lack of response to anti-EGFR therapies has been consistently described. Indeed, K-RAS mutational investigations are now routinely performed in molecular pathology laboratories and recommended for patients with mCRC to determine their potential benefit from anti-EGFR therapies. In our study, all patients with a K-RAS mutation were non-responsive to therapy. Nonetheless, 7 patients with wild-type K-RAS were also found to be non-responders and all of these had high-grade tumor budding. In fact, all patients with an objective response to therapy...
had simultaneous wild-type K-RAS and low-grade tumor budding, thereby improving the predictive accuracy for response from 68% for each biomarker alone to 80% when assessed in combination.

It is recognized that a subgroup of mCRC patients with wild-type K-RAS do not respond to anti-EGFR agents\[15\]. In this study, although all responders were indeed those with wild-type K-RAS and low-grade tumor budding, a considerable proportion of patients, namely 12/43 (27.9%) found with this constellation had PD or SD after treatment. In this setting, we found that loss of PTEN expression could accurately identify 83% of non-responsive cases and that EGFR amplification or copy number gain in PTEN-positive tumors correctly predicted 77% of responders, findings which are in line with numerous reports concerning the predictive value of these markers\[36-38\]. Together, 90.7% (39/43) of patients were correctly classified into response groups using these four features. Mutations in B-RAF and PIK3CA mutations have also been found to lead to non-response in mCRC patients\[39-41\]. Indeed, in this study, cases with either mutation were found to be patients who did not respond to therapy. However, after accounting for K-RAS and tumor budding only 3 B-RAF mutations were observed and 1 PIK3CA mutation was found, therefore the low frequency of these events may have led to their exclusion from the predictive algorithm.

Our study is constrained by several factors, the most important limitation being the sample size. To our knowledge, this is the first study evaluating tumor budding as a potential predictive or prognostic factor in mCRC patients treated with cetuximab or panitumumab, nonetheless these results need to be validated in larger cohorts. Secondly, although tumor budding is considered an additional prognostic factor by the AJCC/UICC, it has not been incorporated into standard pathological routine due to the absence of standardized methods of evaluation. Our cut-off score to define high-grade tumor budding was determined using a 40 × high-power field and found to be reproducible between independent pathologists. Not only was the threshold of 15 tumor buds defined using a valid cut-point determination method and tested using re-sampling methods, but resembles the definition of high-grade tumor budding used by Prall et al\[9\] to define the optimal threshold value for predicting metastatic disease in CRC patients (25 tumor buds observed in the densest region using a 20 × objective lens). Despite these limitations, our study is innovative, in that it appears to be the first evidence suggesting that a histomorphological feature, namely tumor budding, is both a predictive and prognostic factor in patients with mCRC treated with anti-EGFR-based therapies. Moreover, the combined analysis of K-RAS gene status and tumor budding may accurately predict both responders and non-responders with up to 80% accuracy.

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

**COMMENTS**

**Background**

Tumor budding is a histological feature which has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is predictive of both lymph node and distant metastasis stage. Most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer. In addition, tumor budding does not appear to be related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in metastatic colorectal cancer (mCRC) patients treated with anti-epidermal growth factor receptor (EGFR)-based therapies.

**Research frontiers**

Monoclonal antibodies targeting the EGFR such as cetuximab and panitumumab have been recently approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%. Several molecular and protein biomarkers are being investigated as predictive factors of response including K-RAS, B-RAF, PIK3CA and PTEN. The vast majority of studies to date do support a lack of responsiveness in patients with mutation of K-RAS. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

**Innovations and breakthroughs**

The results show that high-grade tumor budding predicts non-response in mCRC patients who receive anti-EGFR therapies. In combination, K-RAS mutation status and tumor budding together can correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable progression-free survival also in a K-RAS-independent manner. This study appears to be the first to show that a histomorphological feature, namely tumor budding, may be a predictive factor of response in mCRC patients treated with anti-EGFR therapy.

**Applications**

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

**Terminology**

Tumor budding is considered the histological hallmark of Epithelial Mesenchymal Transition. Tumor buds are defined as dedifferentiated single cells/small clusters at the invasive front of colorectal cancer.

**Peer review**

This is a well written and presented manuscript. The data are of major importance to the clinicians. The authors studied over more than 40 human samples and made a direct link between molecular expression, prognosis and treatment.

**REFERENCES**

1. Ferlay J, Autier P, Boniol M, Heanue M, Colomet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; 18: 581-592
2. Chung KY, Shia J, Kemeny NE, Shah M, Schwartz GK, Tse A, Hamilton A, Pan D, Schrag D, Schwartz L, Klimenta DS, Fridman D, Kelsen DP, Saltz LB. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005; 23: 1805-1810
3. Cunningham D, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR. Capecitabine and oxaliplatin for advanced esophagogastric cancer. *N Engl J Med* 2008; 358: 36-46
4. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson...
Zlobec I et al. Tumor budding and anti-EGFR therapies

SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008; 26: 1626-1634

5 Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, Siena S, Bardelli A. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 2007; 67: 2643-2648

6 Karapetis CS, Kambath-Ford S, Jonker DJ, O’Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 359: 1757-1765

7 Liévre A, Bacht J, Le Corre D, Boige V, Landi B, Emilie JF, Côté JF, Tomasic G, Penna C, Ducreux M, Rougier P, Saulnier-Llorca F, Laurent-Puig P. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006; 66: 3992-3995

8 Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Hlubek F, Kirchner T. CD8+ lymphocytes/tumour-budding index: a novel independent prognostic marker in pT3 well- or moderately-differentiated rectal carcinoma. Histopathology 2009; 55: 131-139

9 Wang LM, Kevans D, Mulcahy H, O’Sullivan J, Fennelly D, Hyland J, O’Donoghue D, Sheahan K. Tumor budding is a strong and reproducible prognostic marker in TN30 colorectal cancer. Ann Surg Oncol 2009; 33: 134-141

10 Yamauchi H, Togashi K, Kawamura YJ, Horie H, Sasaki J, Saletti P, Romagnani E, Martin V, Molinari F, Karamitopoulou E, Panayiotides I, Karakitsos P, Yoshimura K, Bekku S. Risk factors for an adverse outcome in early invasive colorectal cancer. Gastroenterology 2004; 127: 385-394

11 Prall F, Ostwald C. High-degree tumor budding and p63 expression in sporicolar carcinoma with K-ras gene mutations. Hum Pathol 2007; 38: 1696-1702

12 Ueno H, Morishitsu H, Hashiguchi T, Hatase T, Fujimoto H, Hase K. Predictors of extrahepatic recurrence after resection of colorectal liver metastases. Br J Surg 2004; 91: 327-333

13 Ueno H, Morishitsu H, Hashiguchi T, Shimazaki H, Aida S, Hase K, Matsuoka S, Kanai T, Kurihara H, Ozawa K, Yoshimura K, Bekku S. Risk factors for an adverse outcome in early invasive colorectal cancer. Gastroenterology 2004; 127: 385-394

14 Prall F, Murphy J, Jass JR, Morishitsu H, Talbot IC. Tumor ‘budding’ as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 2002; 40: 127-132

15 Wang LM, Kevans D, Mulcahy H, O’Sullivan J, Fennelly D, Hyland J, O’Donoghue D, Sheahan K. Tumor budding is a strong and reproducible prognostic marker in TN30 colorectal cancer. Ann Surg Oncol 2009; 33: 134-141

16 Compton C. Prognostic Factors in Cancer. 3rd ed. New York: John Wiley & Sons, Inc., 2006

17 Lugli A, Karaimpitoupolou E, Panayiotides I, Karakitsos P, Pallis G, Pellas G, Iezzi G, Spagnoli G, Biel M, Terracciano L, Zlobec I. CD8+ lymphocytes/tumor-budding index: an independent prognostic factor representing a ‘pro-/anti-tumor’ approach to tumor-host interaction in colorectal cancer. Br J Cancer 2009; 101: 1382-1392

18 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92: 205-216

19 Prall F, Murphy J, Jass JR, Morishitsu H, Talbot IC. Tumor ‘budding’ as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 2002; 40: 127-132

20 Compton C. Prognostic Factors in Cancer. 3rd ed. New York: John Wiley & Sons, Inc., 2006

21 Lugli A, Karaimpitoupolou E, Panayiotides I, Karakitsos P, Pallis G, Pellas G, Iezzi G, Spagnoli G, Biel M, Terracciano L, Zlobec I. CD8+ lymphocytes/tumor-budding index: an independent prognostic factor representing a ‘pro-/anti-tumor’ approach to tumor-host interaction in colorectal cancer. Br J Cancer 2009; 101: 1382-1392

22 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92: 205-216

23 Compton C. Prognostic Factors in Cancer. 3rd ed. New York: John Wiley & Sons, Inc., 2006
regulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol* 2009; 20: 84-90

38 **Personeni N**, Fieuws S, Plessevaux H, De Hertogh G, De Schutter J, Biesmans B, De Roock W, Capoen A, Debier-Rychter M, Van Laethem JL, Peeters M, Humbert Y, Van Cutsem E, Tejpar S. Clinical usefulness of EGFR gene copy number as a predictive marker in colorectal cancer patients treated with cetuximab: a fluorescent in situ hybridization study. *Clin Cancer Res* 2008; 14: 5869-5876

39 **Di Nicolantonio F**, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer.

40 **Jhawer M**, Goel S, Wilson AJ, Montagna C, Ling YH, Byun DS, Nasser S, Arango D, Shin J, Klampfer L, Augenlicht LH, Perez-Soler R, Mariadason JM. PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor cetuximab. *Cancer Res* 2008; 68: 1953-1961

41 **Sartore-Bianchi A**, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, Di Nicolantonio F, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res* 2009; 69: 1851-1857

S- Editor Tian L  L- Editor Webster JR  E- Editor Lin YP