Benefit from anti-EGFRs in RAS and BRAF wild-type metastatic transverse colon cancer: a clinical and molecular proof of concept study

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ABSTRACT
Objective Primary tumour location is regarded as a reliable surrogate of colorectal cancer biology. Sensitivity to anti-EGFRs (Epidermal Growth Factor Receptor) of metastatic transverse colon cancers (mTCCs) has usually been assumed similar to right-sided tumours; however, evidence about the clinical behaviour of mTCC is limited. Thus, to verify sensitivity of mTCC to anti-EGFRs we conducted the present study.

Methods Patients with RAS/BRAF wild-type microsatellite stable (MSS) mTCC receiving anti-EGFR monotherapy, or in combination with irinotecan if clearly irinotecan-refractory, were included. Hypothesising an overall response rate (ORR) of 35%, 11 patients, of whom at least 3 were responders, were necessary to be able to reject the null hypothesis of an ORR of 5%, with α and β errors of 0.05 and 0.20. PRESSING panel and consensus molecular subtypes (CMS) were assessed on tumour samples, whereas in-silico data were obtained from TCGA dataset.

Results Among nine eligible patients, four and three achieved response and disease stabilisation (ORR 44%). At a median follow-up of 23.1 months, median progression-free survival and overall survival were 7.3 (95% CI 3.9 to NA) and 15.0 months (95% CI 10.0 to NA), respectively. A MET amplification and an ERBB4 S303F substitution were detected in patients with rapid disease progression, while others had PRESSING panel-negative tumours with CMS2 or CMS4 subtypes.

Conclusions RAS/BRAF wild-type MSS mTCCs may be sensitive to anti-EGFRs, as confirmed by molecular analyses.

INTRODUCTION
Nowadays, patients with RAS and BRAF wild-type metastatic colorectal cancer (mCRC) are the most appropriate candidates for anti-EGFR (Epidermal Growth Factor Receptor)-based treatment.1–3 While right-sidedness has a well-established negative prognostic impact,4 it may also predict resistance to anti-EGFR agents, being a reliable surrogate marker of a complex molecular landscape of negative predictors of benefit from such agents,5 but not from antiangiogenic ones.6,7

What is already known about this subject?
► Primary tumour sidedness (right vs left) has recently entered the therapeutic algorithm for the choice of the first-line treatment of metastatic colorectal cancer (mCRC).
► Tumours originating from the transverse colon are rare and even if they have been included among right-sided tumours in the vast majority of analyses, it is not completely clear if they show more similarities with right-sided or left-sided ones.
► Their sensitivity to anti-EGFRs is not elucidated.

What does this study add?
► Based on an a priori statistical hypothesis, anti-EGFR agents were active in patients with RAS and BRAF wild-type, microsatellite stable (MSS) transverse mCRC.
► Genetic determinants of intrinsic resistance were found in patients with rapid disease progression while signatures potentially related with sensitivity to anti-EGFRs were found in the others.
► Consistent data about CMS distribution were found in TCGA.

How might this impact on clinical practice?
► RAS and BRAF wild-type MSS metastatic transverse colon cancers (mTCCs) seem to differ from tumours originating from caecum, ascending colon or hepatic flexure in terms of primary refractoriness to anti-EGFRs.
► If these results are confirmed by further validation, anti-EGFR-containing regimens may be considered among other appropriate first-line options for patients with mTCC.
Indeed, based on several retrospective data\textsuperscript{8} and posthoc analyses of pivotal randomised clinical trials,\textsuperscript{9-12} primary tumour sidedness entered the therapeutic algorithm for the choice of the first-line treatment: while anti-EGFR-based treatments are the first choice for left-sided RAS and BRAF wild-type tumours, bevacizumab-based chemotherapy combinations and in particular with the intensified FOLFOXIRI regimen,\textsuperscript{13,14} are the preferred options for patients with right-sided primary tumours according to the most recent international guidelines\textsuperscript{15} and clinical recommendations.\textsuperscript{16}

According to the definition adopted in most clinical trials, right-sided and left-sided primary tumours are defined as those originating proximally or distally to the splenic flexure, based on the different embryological origin from the midgut and hindgut, respectively. However, increasing molecular evidence suggests that a \textit{continuum} of genetic characteristics and gene expression profiles can be described throughout different colorectal segments from caecum to extraperitoneal rectum, rather than a simplistic dichotomic distinction between right-sided and left-sided tumours. In particular, genetic markers of primary resistance to anti-EGFRs and gene expression profiles probably associated to limited benefit from these drugs are increasingly prevalent from the rectum to the caecum.\textsuperscript{17-20}

Tumours originating from transverse colon are rare and even if they have been included among right-sided tumours in the vast majority of analyses, it is not completely clear if they show more similarities with right-sided or left-sided ones, so that patients with transverse colon tumours were excluded by the hallmark posthoc analysis of the phase III head-to-head CALGB80405 trial.\textsuperscript{12}

Drawing from these considerations, we assessed the activity of anti-EGFRs in patients with RAS and BRAF wild-type metastatic transverse colon cancer (mTCC). A prospective statistical hypothesis was planned to verify whether the use of anti-EGFRs was supported in this subgroup based on a predefined threshold of clinical relevance. In order to unveil the molecular determinants of our findings, a panel of resistance mechanisms beyond RAS and BRAF mutations, the PRESSING panel,\textsuperscript{19} and consensus molecular subtypes (CMS) were analysed both in tissue samples from treated patients and in-silico.

**Molecular analyses**

Genomic alterations included in the PRESSING panel were investigated as previously reported,\textsuperscript{19} thus exploring several uncommon anti-EGFR resistance mechanisms beyond RAS and BRAF mutations (ie, HER2 amplification/activating mutations; MET amplification; NTRK/ROS1/ALK/RET rearrangements; PIK3CA exon 20 mutations, Pten inactivating mutations, AKT1 mutations). Microsatellite instability (MSI) status was assessed by multiplex PCR as previously described.\textsuperscript{22}

For gene expression analysis, total RNA was extracted from formalin-fixed, paraffin embedded primary tumour samples (Ambion RecoverAll kit as per manufacturers’ instructions). When the primary tumour sample was not available, RNA was extracted from metastatic deposits. Areas with high tumour content were marked by a trained pathologist on H&E slides and macrodissected in five unstained slides. RNA concentration was quantified with NanoDrop 2000 Spectrophotometer (Thermo Fisher). The expression of 38 CMS-subtype specific genes and 10 housekeeping genes was assessed using the nCounter Analysis System (NanoString Technologies) and total RNA in the region of 100 ng according to previously described low-cost protocol.\textsuperscript{23} Each sample was assigned to a CMS subtype using a previously validated single-sample prediction method.\textsuperscript{24}

**TCGA data analysis**

We downloaded single nucleotide variants (SNVs) and clinical data for colon adenocarcinoma and rectum adenocarcinoma from the GDC Legacy Archive.\textsuperscript{25} Processed data (log2-ratio) for ERBB2 and MET somatic copy number alterations were downloaded from cBio Portal.\textsuperscript{26,27} CMS classification for 320 cases was retrieved from Guinney \textit{et al.}\textsuperscript{20} CMS for additional 27 cases was estimated by CMS classifier.\textsuperscript{28} Data about fusion genes involving ALK/ROS/NTRK were retrieved from Pietrantonio \textit{et al.}\textsuperscript{22} TCGA samples with positive pressing panel were defined as those demonstrating at least one of the following events: (1) a non-silent SNVs in \textit{PTEN} (exons 3–8) or \textit{AKT1}, or presence of exon 20 \textit{PIK3CA} mutations; (2) \textit{ERBB2} hotspot mutations as reported in the literature;\textsuperscript{29} (3) presence of \textit{ALK/ROS/NTRK} gene fusions; (4) more or equal than four copies (log2-ratio≥2) for \textit{ERBB2}
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Figure 1  Consort diagram of the study depicting the process of patients’ selection. A total of 401 patients with RAS and BRAF wild-type mCRC treated with an anti-EGFR containing regimen were reviewed. After screening for primary tumour location, 24 patients with mTCC were identified, of whom 9 received an anti-EGFR as single agent or in combination with irinotecan if clearly irinotecan-refractory and were included. mCRC, metastatic colorectal cancer; mTCC, metastatic transverse colon cancer.

and MET. Statistical analysis was performed by custom R scripts.30

Statistical design and analyses
The primary objective of the study was to assess the activity of anti-EGFRs in RAS and BRAF wild-type mTCC. Hypothesising an overall response rate (ORR) of 35% according to RECIST 1.1, deemed as clinically relevant, 11 patients, of whom at least 3 were responders, were needed to be able to reject the null hypothesis of an ORR of 5%, with α and β errors of 0.05 and 0.20.

Disease control rate (DCR) was defined as the percentage of patients achieving complete response (CR), partial response (PR) or stable disease (SD) according to RECIST 1.1. Progression-free survival (PFS) was defined as the time from the beginning of the anti-EGFR treatment to the radiological evidence of disease progression or last follow-up. Overall survival (OS) was defined as the time from the beginning of anti-EGFR treatment to death or last follow-up. PFS and OS analyses were estimated according to the Kaplan-Meier method.

RESULTS
Study population
The flow of patients’ selection is shown in figure 1. Data from 401 patients with RAS and BRAF wild-type mCRC treated with an anti-EGFR containing regimen were reviewed. Among them, 24 patients with mTCC were identified, and 9 of them had received an anti-EGFR as single agent or in combination with irinotecan if clearly irinotecan-refractory and were included. mCRC, metastatic colorectal cancer; mTCC, metastatic transverse colon cancer.

Clinical outcome of patients with RAS and BRAF wild-type mTCC treated with anti-EGFR agents
One CR, three PRs, three SDs and two disease progressions (PD) were reported as best responses to anti-EGFR based treatment, with an ORR of 44% (95% CI 19% to 73%) and a DCR of 78% (95% CI 44% to 95%).

At a median follow-up of 23.1 months, median PFS was 7.3 months (95% CI 3.9 to NA) (figure 2, panel A) and median OS was 15.0 months (95% CI 10.0 to NA) (figure 2, panel B). The treatment history of patients with clinical benefit is graphically depicted in online supplementary file 1. Median treatment duration was
Table 1 Patients and disease characteristics

| Characteristics | Study population | N=9 |
|-----------------|------------------|-----|
| Age (years)     | Median           | 54  |
|                 | Range min-max    | 34–87|
| Gender          | Male             | 3   |
|                 | Female           | 6   |
| ECOG PS         | 0                | 6   |
|                 | 1                | 3   |
| Primary tumour  | No               | 1   |
| resection       | Yes              | 8   |
| Metastatic sites (N) | 1           | 5   |
|                 | >1               | 4   |
| Synchronous mets | No             | 4   |
| Line of anti-EGFR | 1–2            | 3   |
| tx (N)          | >2               | 6   |
| Regimen         | Panitumumab      | 5   |
|                 | Panitumumab plus | 2   |
|                 | irinotecan       | 2   |
|                 | Cetuximab plus   | 2   |
|                 | irinotecan       |     |

**Mets, metastases; tx, treatment.**

5.1 months (95% CI 3.3 to 8.8). Reasons for treatment discontinuation were disease progression in six patients and surgery of residual disease in one patient, while treatment was ongoing at the time of data cut-off (May 2018) in two cases.

**Molecular make-up of patients with RAS and BRAF wild-type mTCC**

Primary tumour samples were available for both PRESSING panel analysis and CMS subtyping (table 2). Regarding the two patients with progressive disease at the first CT scan reassessment, in one case MET amplification by bright-field in situ hybridisation was reported and ERBB4 S303F substitution, reasonably related to the activation of alternative pathways other than EGFR, was found by next generation sequencing in the other case. All other seven patients with clinical benefit from anti-EGFRs had PRESSING panel-negative tumours and CMS2 or CMS4 subtypes.

**TCGA validation analysis**

In TCGA, a total of 335 records of RAS and BRAF wild-type tumours with available information about primary anatomical location and PRESSING panel determinants were retrieved, including 250, 92 and 13 left, right and transverse colorectal primary tumours, respectively. As detailed in figure 3A, determinants of resistance to anti-EGFRs included in the PRESSING panel were found in 25 (10%) out of 250 left-sided, 7 (8%) out of 92 right-sided and 2 (15%) out of 13 transverse colon samples (the latter being represented by one PTEN c.389G>A substitution and one HER2 amplification, respectively). Finally, a total of 173 records of RAS and BRAF wild-type tumours with available information about primary anatomical location and CMS subtypes were retrieved (figure 3B), including 123, 43 and 7 left, right and transverse colorectal primary tumours. Notably, among seven available transverse colon samples, no CMS1 and CMS3 subtypes were detected.

**DISCUSSION**

The concept of sidedness has recently entered clinical recommendations for the choice of the first-line therapy of mCRC based on its value as a surrogate marker of a complex landscape of molecular differences between cancers originating proximally or distally to the splenic flexure with meaningful clinical implications. However, recent in-depth analyses showed that this dichotomic definition is quite simplistic, being unable to properly recapitulate regional variations in tumour biology. To this

![Figure 2](image-url) **Figure 2** Kaplan-Meier curves for PFS (panel A) and OS (panel B) of patients with mTCC receiving an anti-EGFR based therapy. mTCC, metastatic transverse colon cancer; OS, overall survival; PFS, progression-free survival.
Table 2  Molecular analyses in individual patients with mTCC according to PRESSING panel analysis and CMS subtypes, with corresponding outcomes in terms of RECIST response and progression-free survival

| N° patient | PRESSING panel* | MSI status | CMS status | ORR | PFS, months |
|------------|-----------------|------------|------------|-----|-------------|
| #1         | Negative         | MSS        | CMS1       | PD  | 3.2         |
|            | TP53 EX 7 R248Q;|             |            |     |             |
|            | ERBB4 EX 8 S303F|             |            |     |             |
| #2         | Negative         | MSS        | CMS2       | PR  | 6.1         |
|            | TP53 EX 7 G244V;|             |            |     |             |
|            | STK11 EX 4 G180E|             |            |     |             |
| #3         | Negative         | MSS        | CMS4       | CR  | 13.0+       |
|            | TP53 EX 6 R196STOP;|            |            |     |             |
|            | APC EX 15 E1353STOP|           |            |     |             |
| #4         | Negative         | MSS        | CMS4       | PR  | 5.1+        |
|            | TP53 EX 8 R273H;|             |            |     |             |
|            | ATM EX 12 P604S;|             |            |     |             |
|            | APC EX 15 Q12894STOP|          |            |     |             |
| #5         | Negative         | MSS        | CMS4       | SD  | 11.1        |
|            | TP53 EX 8 R273H;|             |            |     |             |
|            | KDR EX 26 E1126V|             |            |     |             |
| #6         | Negative         | MSS        | CMS2       | SD  | 10.0        |
|            | TP53 EX 10 R342STOP|          |            |     |             |
| #7         | Negative         | MSS        | CMS2       | PR  | 3.9         |
|            | TP53 EX 5 F134L|             |            |     |             |
| #8         | Positive MET amplified | MSS | CMS4 | PD  | 2.9         |
|            | No point mutations|          |            |     |             |
| #9         | Negative         | MSS        | NA         | SD  | 7.3         |
|            | TP53 EX 8 G266R;|             |            |     |             |
|            | CTNNB1 EX 3 S37F|             |            |     |             |

*PRESSING panel results are reported overall (negative vs positive) and all mutations detected by NGS are detailed. CMS, consensus molecular subtypes; CR, complete response; EX, exon; MSI, microsatellite instability; MSS, microsatellite stable; NGS, next generation sequencing; ORR, objective response; PFS, progression-free survival; PR, partial response; SD, stable disease; WT, wild-type.

regard, the CRC classification based on the primary location, that is, the specific colon segment where tumours arise, seems to provide a more accurate snapshot of the underpinning tumour biology. By a clinical perspective, these considerations are especially relevant for cancers arising in the transverse colon, since they were classified as right-sided in most subgroup analyses of randomised trials, but their molecular landscape, based on mutation clustering, seems closer to left-sided tumours.\(^{18}\) As a practical implication, assessing the sensitivity of RAS and BRAF wild-type mTCCs to anti-EGFR monoclonal antibodies is crucial in order to properly build the therapeutic route of these patients from the very beginning, given the substantial impact of the first-line therapy on the whole disease history.

Loree et al.\(^{19}\) faced this issue, retrospectively evaluating the response to anti-EGFR-containing regimens administered in second or further lines to 17 patients affected by RAS and BRAF wild-type mTCCs showing an ORR according to RECIST V1.1 of 35%, with an 82% DCR and a median PFS of 5.9 months. However, all patients had received the targeted agent in combination with at least one cytotoxic drug, thus preventing from drawing conclusions about the actual benefit derived from anti-EGFR monoclonal antibodies alone.

In order to prospectively challenge these suggestive findings, we included in the present analysis only patients treated with an anti-EGFR as monotherapy or in combination with irinotecan in the case of clear irinotecan-refractoriness. Consistently, we limited our investigation to patients with RAS and BRAF wild-type tumours, since they are the only potential candidates to anti-EGFRs. Moreover, given the reasonable association of microsatellite instability with limited benefit from a targeted approach against one single pathway,\(^{19}32\) and the prevalence of microsatellite instability in mTCC being similar to ascending colon and higher than left-sided segments, only patients with RAS and BRAF wild-type MSS tumours were included.

In our series, an ORR of 44% with a 78% DCR was reported, thus outlining a potential role for anti-EGFR agents in the treatment of patients with mTCC. The prespecified hypothesis underlying our analysis was confirmed, since more than three responses were registered even if only 9 patients were included instead of 11, as initially planned. The main limitation of this study is the small sample size, due to the rarity of the transverse location and the choice to include only patients with RAS and BRAF wild-type tumours where the effect of anti-EGFRs was clearly distinguishable without the confounding effect of the associated chemotherapy backbone.

Though acknowledging the limitations of our pragmatic approach, including also the lack of a proper control arm and the review of retrospectively collected data, and therefore the low level of produced evidence, a prospective effort to answer this clinically relevant question through a randomised clinical trial focused on this rare population (RAS and BRAF wild-type, MSS mTCCs) would be hardly feasible. On the other hand, the validation of present findings in posthoc analyses of randomised trials with available information about the precise anatomical location would be highly desirable.

By a molecular perspective, heterogeneous features were reported in analysed samples and potential mechanisms of intrinsic resistance to anti-EGFRs were found in both cases of rapid disease progression. Also with regard to CMS classification, most cases presented potentially EGFR-dependent signatures, as confirmed also by the in silico validation that took advantage of the small number of RAS and BRAF wild-type MSS mTCCs included in the TCGA database.
Besides the identified molecular alterations underpinning primary resistance to anti-EGFRs, this could be also sustained by RAS mutations not recognised by conventional tissue analyses at baseline, that could have been detected in circulating tumour DNA through liquid biopsies.33

Based on our results, RAS and BRAF wild-type MSS mTCCs seem to differ from tumours originating from caecum, ascending colon or hepatic flexure in terms of primary refractoriness to anti-EGFRs. Therefore, if these results are confirmed by further validation, anti-EGFR-containing regimens may be considered among other appropriate first-line options for patients with mTCC. The evaluation of a panel of molecular mechanisms of intrinsic resistance in RAS and BRAF wild-type tumours, that is, the mentioned PRESSING panel,19 could allow performing a negative hyperselection of resistant patients, in the absence of positive predictors of benefit from anti-EGFR agents.

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