Intratumoral Transcriptome Heterogeneity Is Associated With Patient Prognosis and Sidedness in Patients With Colorectal Cancer Treated With Anti-EGFR Therapy From the CO.20 Trial

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PURPOSE Metastatic colorectal cancers (mCRCs) assigned to the transit-amplifying (TA) CRCAssigner subtype are more sensitive to anti–epidermal growth factor receptor (EGFR) therapy. We evaluated the association between the intratumoral presence of TA signature (TA-high/TA-low, dubbed as TA-ness classification) and outcomes in CRCs treated with anti-EGFR therapy.

PATIENTS AND METHODS The TA-ness classes were defined in a discovery cohort (n = 84) and independently validated in a clinical trial (CO.20; cetuximab monotherapy arm; n = 121) and other samples using an established NanoString-based gene expression assay. Progression-free survival (PFS), overall survival (OS), and disease control rate (DCR) according to TA-ness classification were assessed by univariate and multivariate analyses.

RESULTS The TA-ness was measured in 772 samples from 712 patients. Patients (treated with anti-EGFR therapy) with TA-high tumors had significantly longer PFS (discovery hazard ratio [HR], 0.40; 95% CI, 0.25 to 0.64; P = .001; validation HR, 0.65; 95% CI, 0.45 to 0.93; P = .018), longer OS (discovery HR, 0.48; 95% CI, 0.29 to 0.78; P = .003; validation HR, 0.67; 95% CI, 0.46 to 0.98; P = .04), and higher DCR (discovery odds ratio [OR]; 14.8; 95% CI, 4.30 to 59.54; P < .001; validation OR, 4.35; 95% CI, 2.00 to 9.09; P < .001). TA-ness classification and its association with anti-EGFR therapy outcomes were further confirmed using publicly available data (n = 80) from metastatic samples (PFS P < .001) and patient-derived xenografts (P = .042). In an exploratory analysis of 55 patients with RAS/BRAF wild-type and left-sided tumors, TA-high class was significantly associated with longer progression-free survival and trend toward higher response rates (PFS HR, 0.53; 95% CI, 0.28 to 1.00; P = .049; OR, 5.88; 95% CI, 0.71 to 4.55; P = .09; response rate 33% in TA-high and 7.7% in TA-low).

CONCLUSION TA-ness classification is associated with prognosis in patients with mCRC treated with anti-EGFR therapy and may further help understanding the value of sidedness in patients with RAS/BRAF wild-type tumors.

INTRODUCTION Epidermal growth factor receptor (EGFR)-targeting antibodies cetuximab and panitumumab are available treatment options for approximately 40% of patients with metastatic colorectal cancer (mCRC).1 Patient selection based on RAS and BRAF wild-type status and sidedness has improved overall response rates and survival outcomes. Nevertheless, 30%-60% of eligible patients do not benefit from these expensive drugs.2-4 As a shift from the traditional paradigm of negative molecular selection, we previously demonstrated that the transit-amplifying (TA) CRCAssigner (CRCA) subtype was enriched for cetuximab-responsive tumors,5 a finding independently validated in a clinical study,6 in a panel of CRC xenografts5 and cell lines.5,7 However, responses were also seen in other groups, such as the poorly differentiated stem-like subtype,5,7 albeit at a lower frequency. This suggested a scope for refining a previously validated gene-expression–based classifier to assess anti-EGFR therapy response in CRC.
**CONTEXT**

**Key Objective**
To evaluate whether the presence of the transit-amplifying (TA) subtype gene signature (dubbed as TA-ness classification) representing the intratumoral transcriptome heterogeneity is associated with anti–epidermal growth factor receptor (EGFR) therapy outcomes.

**Knowledge Generated**
The TA-ness classification is an easily detectable biomarker of intratumoral transcriptome heterogeneity, which was retrospectively evaluated in 712 patient samples, including those from a clinical (CO.20) trial, which showed prognostic significance in patients treated with anti-EGFR therapy. This biomarker provides additional biologic insights for the association between RAS/BRAF wild-type left-sided tumors (enriched for TA-high) and anti-EGFR therapy benefit.

**Relevance**
With further validation, TA-ness may represent a positive selection biomarker for patients with RAS/BRAF wild-type left-sided metastatic colorectal cancer who are most likely to benefit from anti-EGFR therapy.

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TA subtype tumors are characterized by gene signatures similar to normal TA cells of the colonic crypt, that is, those in transit between stem cells in the crypt base and differentiated cells at the top of the crypt.⁸ After asymmetric division, stem cells generate rapidly proliferating TA cells characterized by increased EGFR expression that eventually differentiate into goblet cells and enterocytes.⁸,⁹ We evaluated a hypothesis that tumors with increased TA gene signature expression (irrespective of TA or other subtypes) may be associated with anti-EGFR therapy outcomes. This may capture intratumoral transcriptomic heterogeneity in CRCs with more than one subtype signature coexisting in the same tumor and improve assessment of prognosis and its association with RAS/BRAF wild-type statuses and tumor sidedness in patients with mCRC treated with anti-EGFR therapy.¹⁰

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**PATIENTS AND METHODS**

**Study Population**

Four independent cohorts of patients with CRC treated with anti-EGFR therapy (n = 315) were examined: one discovery and three validation (two clinical and one experimental) cohorts (Fig 1). The discovery cohort included chemorrefractory patients (n = 84) who had received anti-EGFR therapy as a single agent or in combination with chemotherapy after progression while receiving irinotecan (during or within 3 months from the end of treatment) as part of standard treatment at the Royal Marsden Hospital (RMH; n = 59; United Kingdom, ethics committee: 10/H0308/28; and ClinicalTrials.gov identifier: NCT02112357) or within the context of a case-control study in Italian institutions (PRESSING, n = 25; ethics committee Area Vasta Nord Ovest number 1333/17²). All patients signed an informed consent for translational research and received at least one cycle of anti-EGFR therapy. Nineteen and 12 patients from the RMH cohort were treated before the implementation of KRAS testing (August 2009) and extended RAS testing (December 2011), respectively.¹¹,¹² All patient samples from the PRESSING study had extended RAS/BRAF wild-type tumors.

One of the clinical validation cohorts included 121 patients with KRAS exon 2 wild-type tumors who had received single-agent cetuximab within the control arm of the CO.20 phase III randomized clinical trial (ClinicalTrials.gov identifier: NCT00640471).¹³ This correlative analysis was approved by the Joint Canadian Cancer Trial Group and Australasian Gastrointestinal Trial Group (CCTG/AGITG) Correlative Sciences and Tumor Biology Committee.

Two additional public gene expression datasets (n = 397; not treated with anti-EGFR therapy) of primary CRC samples (GSE39582; n = 328) and liver mCRC lesions (GSE73255; n = 69) were evaluated.¹⁴,¹⁵ Only samples with known KRAS wild-type status were selected.

**Nucleic Acids Extraction**

Formalin-fixed paraffin-embedded (FFPE) tissues were evaluated by a trained pathologist; areas with at least 30% of tumor content were marked on hematoxylin and eosin slides and macrodissected in unstained slides (7- to 10-µm thickness). After deparaffinization, total RNA and DNA were simultaneously isolated using the Ambion RecoverAll kit (discovery) or QIAamp nucleic acid FFPE tissue kit (validation) and quantified with NanoDrop 2000 Spectrophotometer (Thermo Fisher, Waltham, MA) according to the manufacturer’s instructions. The DNA quantification (validation) was performed using a PICO plate reader and the Qubit dsDNA HS kit (ThermoFisher), with an 8-point reference curve.

**Biomarker Assessment**

Thirty-eight published CRCAssigner subtype-specific genes (CRCA-38) were assessed using the NanoString platform (NanoString Technologies, Seattle, WA) according to a previously validated custom CRC subtype-based gene expression analysis assay.¹⁶ Based on the correlation coefficient values after Pearson correlation analysis between...
Discovery cohort
Retrospective tissue collections

- RMT cohort (n = 30)
- Included because of low tumor content in the block or low RNA conc. (n = 28)
- Samples with quality RNA (n = 18)
- Patients for final analysis (n = 6)
- Patients with RNA (n = 11)
- Patients with O2-Flag after iCorr analysis (n = 10)
- Patients with O2-Flag after iCorr analysis (n = 5)
- Patients with O2-Flag after iCorr analysis (n = 4)
- Patients for final analysis (n = 2)

Validation cohort 1
Prospectively enrolled in the CO.20 study

- Patients (out of 278 in cetuximab arm) (n = 160)
- Patients included because of low tumor content in the block or low RNA conc. (n = 17)
- Patients with O2-Flag after iCorr analysis (n = 10)
- Patients with O2-Flag after iCorr analysis (n = 9)
- Patients with O2-Flag after iCorr analysis (n = 6)
- Patients for final analysis (n = 3)

Validation cohort 2
Khanmata-Ford dataset (published)

- Gene expression from liver metastases of patients treated with oxaliplatin (n = 80)
- Unresectable PDX (control; n = 30)
- PDXs treated with oxaliplatin (n = 10)

Outcome
Cohort
TA-ness
No.
Median
PFS
(months)
Univariate
analysis
hazard ratio (95% CI)
Log-rank
P
Multivariate
analysis
hazard ratio (95% CI)
Cox regression
P

PFS
Discovery
TA-high
92
5.73
0.46
(2.26 to 5.64)
< .001
0.46
(0.29 to 0.70)
.01
TA-low
32
2.22
0.55
(0.85 to 1.30)
< .05
0.65
(0.42 to 0.98)
.02

PFS
Validation
TA-high
45
2.03
0.55
(1.05 to 2.00)
< .05
0.50
(0.37 to 0.96)
.024
TA-low
11
1.94
0.55
(0.85 to 1.00)
< .05
0.50
(0.37 to 0.96)
.024

PFS
RAS/BRAF
mutated
dataset
TA-high
42
5.02
0.53
(2.15 to 1.00)
< .05
0.50
(0.37 to 0.96)
.024
TA-low
13
2.12
0.55
(0.85 to 1.00)
< .05
0.50
(0.37 to 0.96)
.024

RESULTS

Retrospective anti–EGFR-treated tumor samples from 205 patients were identified from the discovery and validation (CO.20) cohorts after clinical review and quality control of the tumor blocks and tumor-derived RNA (Fig 1A). Eighty-four patients formed the discovery cohort, and 121 patients from the CO.20 study formed the primary validation cohort (Data Supplement). These cohorts were analyzed for TA-ness classification using our subtype-based published CRCA gene expression assay. Moreover, an experiment

Statistical Analysis

Progression-free survival (PFS) was the primary endpoint. Overall survival (OS), disease control rate (DCR), and response rate were secondary endpoints. Kaplan-Meier survival function was used to estimate survival curves followed by log-rank test to analyze differences in survival time. Fisher’s exact test was used to compare categorical variables, and Wilcoxon signed rank test with $P < .05$ was used to assess the association between TA-ness classes and percentage of tumor shrinkage (using RECIST) criteria in a subgroup of the discovery cohort. Multivariate analyses were performed for the discovery and the validation cohorts, using Cox proportional hazard regression models with 95% CIs. An ROC curve was built to evaluate the accuracy of TA-ness signature and sidedness in defining anti-EGFR clinical benefit. Although the statistical analysis of discovery cohort was performed by the Institute of Cancer Research statistician, the validation cohort was independently analyzed by CCTG/AGITG investigators blinded to the biomarker cut-off analysis. Additional methods are available in the Data Supplement.

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In the discovery cohort, the TA-ness classification was assessed using samples from primary tumors in 76% of patients and samples from metastatic sites in 24% of patients. Nevertheless, the origin of diagnostic samples did not affect the classification (Data Supplement). To further confirm that the association was independent of the diagnostic sample of origin and to further validate the results, we examined the Khambata-Ford publicly available (microarray) dataset \(^{17}\) of mCRC samples from patients treated with cetuximab. Similar to the discovery and validation cohorts, TA-high class was significantly associated with longer PFS (HR, 0.36; 95% CI, 0.22 to 0.57; \(P < .001\)) in the Khambata-Ford data (Fig 2E). To further confirm that the TA-ness can be assessed in both primary tumors and metastatic lesions, \(KRAS\) wild-type samples from two publicly available datasets\(^{14,15}\) were selected; 328 primary tumors and 69 liver metastases were classified into TA-high and TA-low. Similar distribution of the two classes was demonstrated (Fig 3C).

Beyond \(RAS/BRAF\) mutational status, sidedness is a recognized selection factor for anti-EGFR therapy benefit: patients with left-sided tumors benefit more than patients with right-sided tumors.\(^{4}\) However, the biology behind this association remains unclear. First, we further confirmed significant association (\(P < .001\)) between TA-ness classification and sidedness in \(KRAS\) wild-type primary tumors (GSE39582; Fig 3D). Then, we sought to discover whether the TA-ness classification could further refine the selection of patients in addition to \(RAS/BRAF\) status and sidedness. Within discovery and validation cohorts (\(n = 205\)), high-sensitivity next-generation sequencing \(RAS/BRAF\) mutational analysis was available for 118 patients: 71 were classified as \(RAS/BRAF\) wild-type, of which 53 were assigned to TA-high (75%) class. The accuracy of the classification (measured as AUC) appeared higher than the accuracy of the sidedness in defining DCR (AUC, 0.70 vs 0.59; Data Supplement), which warrants additional validation.

Among 55 patients with \(RAS/BRAF\) wild-type and left-sided tumors (the population that nowadays would meet the clinical selection criteria for anti-EGFR therapy), the median PFS of TA-high left-sided tumors was significantly longer than that of TA-low left-sided tumors (5.62 vs 2 months; \(HR, 0.53; 95\% \text{ CI}, 0.28 \text{ to } 1.00; P = .049\); Fig 2F). The response rate is 33% in TA-high and 7.7% in TA-low (Data Supplement).

**DISCUSSION**

In this study, we explored, for the first time (to our knowledge), a proof-of-concept intratumoral heterogeneity-based transcriptome biomarker of prognosis and potential response in patients treated with anti-EGFR agents along with the clinically established criteria of \(RAS/BRAF\) wild-type status and tumor sidedness. Two different classes can be identified in patient samples based on the TA-ness class.
Survival (probability)

Time (months)

A. PFS - discovery cohort (n = 84)

HR, 0.40 (95% CI, 0.25 to 0.64)  
$P < .001$

B. PFS - validation cohort (n = 121)

HR, 0.65 (95% CI, 0.48 to 0.93)  
$P < .02$

C. OS - discovery cohort (n = 84)

HR, 0.46 (95% CI, 0.29 to 0.78)  
$P = .003$

D. OS - validation cohort (n = 121)

HR, 0.67 (95% CI, 0.46 to 0.98)  
$P = .04$

E. PFS - Khambata-Ford dataset (n = 80)

HR, 0.36 (95% CI, 0.22 to 0.57)  
$P < .0001$

F. PFS - extended RAS/BRAF left-sided tumors (n = 55)

HR, 0.53 (95% CI, 0.28 to 1.00)  
$P = .049$
Intratumoral Transcriptome Heterogeneity and Anti-EGFR Therapy

**FIG 2.** Kaplan-Meier survival curves of patients with transit-amplifying (TA)-high versus TA-low tumors treated with anti-epidermal growth factor receptor (EGFR) therapy. (A) Progression-free survival (PFS) from discovery cohort (n = 84). (B) PFS from validation CO.20 cohort (n = 121). (C) Overall survival (OS) from discovery cohort (n = 84). (D) OS from validation CO.20 cohort (n = 121). (E) PFS from publicly available Khambata-Ford et al.17 data (n = 80). (F) PFS from extended RAS/BRAF left-sided tumors (n = 55). HR, hazard ratio. P values are from log-rank test.

**FIG 3.** Disease response, change in tumor volume, primary versus metastatic tumors, and sidedness according to transit-amplifying (TA)-ness classification. (A) A waterfall plot showing a subgroup of patients within the discovery cohort (n = 35) showing disease response (treated with anti–epidermal growth factor receptor [EGFR] drug) according to RECIST criteria and TA-ness classification. Mutational status and sidedness are also shown. P values were from Wilcoxon test. (B) A waterfall plot showing change in tumor (percent) volume in anti-EGFR–treated mouse-propagated patient tumor samples (n = 30) compared with matched control treated (baseline; n = 30) mouse-propagated patient tumors. The bars in the graph show TA-ness classification for the matched patient metastatic liver samples (n = 30), and the bars below the graph show the same classification for matched mouse-propagated patient tumors (treated v control). P values were from the Wilcoxon test. (C) A bar plot showing the proportion of TA-ness classes in KRAS wild-type primary colorectal cancer tumors and liver metastases. (D) Heat map showing the association between TA-ness classes and sidedness in KRAS wild-type primary tumors (GSE39582). PDX, patient-derived xenograft.

(intratumoral transcriptome) classification: TA-high and TA-low. This classification has the advantage of providing a qualitative assessment in all the samples, including the non-TA subtypes, overcoming the limitations posed by intratumoral heterogeneity when using the conventional molecular subtyping classification as a potential tool to assess benefit from anti-EGFR therapy. TA-high tumors were significantly and primarily associated with prognosis.
therapy.20 In contrast, we evaluated a re
was validated in a
cetuximab-only
patient concordance was not assessed; therefore, addi-
sample is not available or of poor quality; however, intra-
assessed in metastatic lesions when the primary tumor
vant, because it means that the classi
is challenging; in fact, the negative predictive value of RAS/
mutations was retrospectively demonstrated in
extended RAS/BRAF wild-type tumors. Last, this was a
was designed with an up-front prospective inclusion of
control group, limiting the assessment of a TA-ness biomarker
as prognostic rather than predictive. In current clinical
practice, anti-EGFR therapy is more frequently used in the
first-line rather than the chemorefractory setting. Hence,
the assessment of the TA-ness in more contemporary first-
line trials, including a control arm and with balanced
mutational status between arms, is warranted in the future.

In conclusion, we demonstrated that the detection of the
TA-ness classification in primary CRC or mCRC samples
shows prognostic significance in patients treated with anti-
EGFR therapy and provides an additional biologic expla-
nation for left-sided versus right-sided tumors, which is
currently used for the differential anti-EGFR therapy benefit
in patients.4 Whether the TA-ness classification can be
used as a biomarker to improve patient selection for anti-
EGFR therapy benefit in mCRC warrants additional vali-
dations in the future.

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST
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**APPENDIX**

**FIG A1.** Receiver operating characteristic (ROC) curve to determine the best cut-off to define disease control rate in the discovery cohort. 1° represents samples classified into transit-amplifying (TA) tumor with highest (rank) correlation with CRCAssigner (CRCA)-38 centroids. Similarly, 2° to 5° represents samples classified into TA between second highest to lowest ranks out of five CRCA-38 subtypes. Four different combinations of the ranks (represented in different colors) were tested for disease control rate using ROC. The best combination was that with two groups: 1° to 3° versus 4° and 5°. AUC, area under the curve.
FIG A2. (A) Kaplan-Meier survival curve of patients with transit-amplifying (TA) tumor versus non-TA tumor in the discovery cohort subtyped using conventional subtyping approach. \( P \) represents log-rank test. (B) Receiver operating characteristic curve comparing the accuracy of the TA-ness classification versus sidedness in 71 patients with \textit{RAS/BRAF} wild-type tumors. AUC, area under the curve. PFS, progression-free survival.