Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features

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Background: Differences exist between the proximal and distal colon in terms of developmental origin, exposure to patterning genes, environmental mutagens, and gut flora. Little is known on how these differences may affect mechanisms of tumorigenesis, side-specific therapy response or prognosis. We explored systematic differences in pathway activation and their clinical implications.

Materials and methods: Detailed clinicopathological data for 3045 colon carcinoma patients enrolled in the PETACC3 adjuvant chemotherapy trial were available for analysis. A subset of 1404 samples had molecular data, including gene expression and DNA copy number profiles for 589 and 199 samples, respectively. In addition, 413 colon adenocarcinoma from TCGA collection were also analyzed. Tumor side-effect on anti-epidermal growth factor receptor (EGFR) therapy was assessed in a cohort of 325 metastatic patients. Outcome variables considered were relapse-free survival and survival after relapse (SAR).

Results: Proximal carcinomas were more often mucinous, microsatellite instable (MSI)-high, mutated in key tumorigenic pathways, expressed a B-Raf proto-oncogene, serine/threonine kinase (BRAF)-like and a serrated pathway signature, regardless of histological type. Distal carcinomas were more often chromosome instable and EGFR or human epidermal growth factor receptor 2 (HER2) amplified, and more frequently overexpressed epiregulin. While risk of relapse was not different per side, SAR was much poorer for proximal than for distal stage III carcinomas in a multivariable model including BRAF mutation status [N = 285; HR 1.95, 95% CI (1.6–2.4), P < 0.001]. Only patients with metastases from a distal carcinoma responded to anti-EGFR therapy, in line with the predictions of our pathway enrichment analysis.

Conclusions: Colorectal carcinoma side is associated with differences in key molecular features, some immediately druggable, with important prognostic effects which are maintained in metastatic lesions. Although within side significant differences exist, the clinical impact is likely less pronounced than previously anticipated.

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molecular heterogeneity remains, our findings justify stratification of patients by side for retrospective and prospective analyses of drug efficacy and prognosis.

**Key words:** colon cancer, expression profiling, mutations, oncogenic pathways, survival

**introduction**

Current understanding of molecular mechanisms involved in colorectal cancer (CRC) supports three main molecular pathways. The almost classical chromosomal instability (CIN) pathway is based on the seminal publication of Vogelstein and contains most of the Kirsten rat sarcoma viral oncogene homolog (KRAS) mutated CRCs. The mismatch repair deficient or microsatellite instable (MSI) pathway was discovered through elucidation of the gene mutations responsible for Lynch syndrome and is characterized by a hypermutating state and frequent B-Raf proto-oncogene, serine/threonine kinase (BRAF) V600E mutation. The CpG island methylator phenotype (CIMP) pathway goes along with the occurrence of serrated precursor lesions and is also strongly related to the MSI pathway, notably through frequent methylation of the mutL homolog 1 promoter, which confers MSI-high status [1]. The pathways are sufficiently distinct to be conceptually valid, but they also significantly overlap. This makes the development of new molecular modalities of classification of CRC a complex task [2].

Different approaches toward molecular classification have been undertaken, based on gene expression profiles and the TCGA whole-genome sequencing effort. We and others have proposed gene expression-based molecular subgroups [3–6] that share (groups of) molecular characteristics while maintaining significant intragroup heterogeneity. Typical examples are the segregation of clinically significant subgroups such as those BRAF-mutated or expressing a BRAF-mutated gene expression signature [7] and MSI or expressing an MSI-like signature [8]. Signatures and subgroups identified by them intend to define patient categories for which treatment needs and/or response to treatment may differ.

The systematic attempt toward subclassification is epitomized in the TNM staging approach and stage grouping as its derivative. Anatomic characteristics related to tumor spread still dictate to a large extent, even in this era of molecular scrutiny, how a patient will be treated. Strikingly, tumor side in terms of proximal or distal colon has gained in prominence in recent years. Initially, this was recognized mostly through the strong preference for the proximal colon for cancers associated with the Lynch syndrome. This paved the way toward the recognition that proximal carcinomas are more often MSI, BRAF-mutated and express the CIMP phenotype [9, 10]. This might be related to differences in biology between the proximal and distal colon, with potentially significant impact on tumorigenesis in these respective sides. However, little is known about the mechanisms responsible for such tumor heterogeneity. One distinctive feature is represented by their embryonic derivation, which is the midgut and the hindgut for the proximal and distal colon, respectively. The pathways involved in the development of these segments have been extensively explored and should be taken into consideration when the biology of their derived cancers is considered. Additionally, the differences in luminal content and bacterial flora between the left and right colon may influence oncogenesis [11]. Therefore, tumor location is a major source of biological heterogeneity, potentially with prognostic and predictive implications in view of the fact that the mortality rate is higher in proximal than in distal colon cancer (CC) [12–15].

We hypothesized that the carcinogenic pathway is different between proximal and distal colon tumors, and that this would be reflected in size-associated differences in the molecular characteristics of the tumors. This might have profound prognostic and therapeutic implications. We tested this by comparing clinicopathological and molecular characteristics of carcinomas in the proximal versus distal colon in two large CC cohorts.

**materials and methods**

**patients**

Clinicopathological data were available for a cohort of 3045 CC patients enrolled in the PETACC3 adjuvant chemotherapy trial. A subset of those patients had molecular data (N = 1404), including BRAF, KRAS, and PIK3CA mutation status, MSI status, and 18q arm loss of heterozygosity (LOH). Parallel gene expression (N = 589) and DNA copy number profiles (N = 199) were also available [16, 17]. Clinicopathological (N = 413) and molecular information (somatic mutations N = 199, RNAseq N = 325) for additional CC patients were obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) [18].

Gene expression profiles of 84 normal colon samples were derived from four datasets (TCGA CC, GSE14333, GSE8671, and GSE41258).

To assess tumor side-effect on response to anti-epidermal growth factor receptor (EGFR) therapy, we studied a cohort of 435 chemorefractory metastatic CRC patients [19].

Tumors located in the splenic flexure, descending colon, and sigmoid colon were defined as proximal, while cecum, ascending, and hepatic flexure were classified as distal. Intrapерitoneal rectum and distal rectum were excluded from the analysis. Transverse CCs (for the lack of clarity as to the exact location) were included exclusively when assessing feature distribution along the bowel. Further information is given in supplementary Materials and Methods, available at *Annals of Oncology* online.

**statistical analysis**

Gene expression and copy number data analyses were processed as described elsewhere [3, 16]. Biological interpretation was carried out using tools and signatures described in supplementary Materials and Methods, available at *Annals of Oncology* online. We applied a Bayesian model selection approach to test if variables could be explained better by a flat, dichotomous, or a continuum model of variation along the bowel.

We assessed differences in the distribution of categorical variables with Fisher’s test or Pearson’s χ² test, as indicated. We used the Cox proportional hazards model to assess the association of tumor side with time-to-event end points and Kaplan–Meier method for figures.

**results**

The frequency distribution of the clinicopathological features along the bowel was analyzed using the PETACC3 and TCGA cohorts. Proximal carcinomas were associated with higher age, node-negative stage, high grade, and mucinous differentiation.
(supplementary Table S1 and S2, available at Annals of Oncology online). Furthermore, proximal carcinomas disseminated more often to the abdominal viscera and lymph nodes, whereas distal carcinomas had a higher frequency of liver and chest metastases. Concerning the distributions of the variables along the bowel, including, for this, the transverse colon (supplementary Table S3 and S4, available at Annals of Oncology online) most of them favored a biphasic model, with the exception of MSI in the PETACC3 dataset which showed a gradual distribution. Based on these findings, we explored the molecular bases of such differences starting from the colon normal mucosa.

Gene expression profiles of 84 normal samples (34 proximal and 50 distal) collected from four public datasets were analyzed to assess the effect on gene expression in normal mucosa based on their location. In a meta-analytical approach including colon side as a predictor, we identified 351 genes differentially expressed—157 overexpressed in the proximal and 194 in the distal colon (supplementary Table S5, available at Annals of Oncology online). Notably, the expression of some HOX genes involved in colon development (HOXC6, HOXB6, and HOXB13) as well as of the EGFR ligand epiregulin (EREG) was different according to side. Gene set enrichment analysis using DAVID evidenced that genes overexpressed in the proximal colon were associated with an inflammatory response and drug metabolism (notably of cytochrome P450 superfamily—supplementary Table S6, available at Annals of Oncology online).

Difference in gene expression between proximal and distal tumors was explored in 589 CC samples (211 proximal and 378 distal) from the PETACC3 dataset, using a linear model controlling for potential confounders such as BRAF and KRAS mutation status and MSI. After correction for multiple testing, 576 genes were found differentially expressed (158 genes up-regulated in proximal and 418 in distal carcinomas—supplementary Table S7, available at Annals of Oncology online), showing mainly a biphasic midgut/hindgut pattern, as for the clinicopathological features. Overall, gene expression fold-changes between the two sides were small in magnitude.

Only 20 genes (including two HOX genes—HOXC6 and HOXB13) were found to be in common with the 351 genes found differentially expressed in the normal colon. Notably, within the group of BRAF-mutated carcinomas (which are mostly proximal), no differences were found between proximal and distal carcinomas (data not shown).

To elucidate if tumor side influences the type of pathways exploited by tumor cells to promote and sustain CC tumorigenesis, we selected a set of gene signatures representing the main biological processes involved in CC (details in supplementary Table S8, available at Annals of Oncology online). The level of those signatures was compared between sides in 589 CC from the PETACC3 dataset and 325 from the TCGA dataset and results combined meta-analytically. Figure 1 summarizes the strength and direction of the association between proximal and distal CC.

**Figure 1.** Barplot showing signed statistic of the association between gene signatures and tumor side observed in 589 CRC from the PETACC3 and 325 from the TCGA datasets. (A) The analysis was carried out considering all the patients, (B) or focusing on MSS, BRAF, and KRAS wild-type tumors. Association of the gene signatures with tumor location was assessed separately within each dataset using a linear model. Results were combined using Fisher’s method. Blue bars represent levels of significance after adjustment for multiple testing [P < 0.05 after Bonferroni correction for all patients (A) and false discovery rate (FDR) < 0.25 after Benjamini–Hochberg procedure when considering MSS, BRAF, and KRAS wild-type tumors (B)].
the signatures and tumor side considering all samples or focusing exclusively on microsatellite stable (MSS), BRAF, and KRAS wild-type patients. BRAF-like, MSI-like, and serrated adenoma signatures showed the strongest bias between sides, suggesting that these are the most prevalent signatures distinguishing proximal from distal tumors. Notably, this difference is also observed in the set of MSS, KRAS, and BRAF wild-type tumors (supplementary Figure S1, available at Annals of Oncology online). In the whole patient cohort, we also found a significant positive association between proximal tumors and T-cell activation, JAK-STAT, angiogenesis, apoptosis, RAS, and mitogen-activated protein kinase (MAPK) activation. In contrast, distal carcinomas were associated with WNT, MYC, and SCR activation as well as the presence of intestinal stem cells. Notably, distal MSS and BRAF and KRAS wild-type carcinomas were also associated with human epidermal growth factor receptor 2 (HER2) and EGFR activation signaling, which parallels the observation that EREG (EGF ligand) was among the most overexpressed genes in distal carcinomas.

Copy number variation (CNV) analysis was carried out on a subset of 199 patients (127 distal and 72 proximal) from the PETACC3 study. Distal carcinomas showed a significantly higher proportion of CNV+ patients (57%) than proximal carcinomas (40%) ($\chi^2$ test, $P = 0.029$), as well as a higher number of amplification/deletions (supplementary Figure S2, available at Annals of Oncology online). Regions on chromosomes 10, 11, 14, 18, and 20 were altered with different frequency (supplementary Table S9 and Figure S3 and S4, available at Annals of Oncology online). Notably, gain of 20q and loss of 18q were found significantly more often in distal carcinomas (supplementary Figure S2 and Table S1, available at Annals of Oncology online), which corroborate overexpressed in distal tumors of a significant proportion (20%) of genes located on 20q (Fisher’s test, $P < 0.0001$).

Chromosomal regions hosting receptor tyrosine kinases were more often amplified in distal (60/127, 47%) than in proximal (23/72; 32%) carcinomas, including the ErbB family members HER2 and EGFR (16/127 versus 1/72, Fisher’s test $P < 0.001$; supplementary Figure S5, available at Annals of Oncology online). Mutation frequency was analyzed in 199 tumors (78 distal and 121 proximal) from the TCGA CC collection. As previously described [18], mutations were more frequent in MSI-high than in MSS carcinomas (data not shown). However, in proximal MSS carcinomas, the number of deleterious mutations was higher than in distal MSS carcinomas (supplementary Figure S6, available at Annals of Oncology online), even after removing all hypermutant tumors (non-silent mutation rate >450). A similar trend was also observed when considering only oncogenes, indicating that the higher mutation rate was potentially an important feature of proximal tumors beyond the MSI/hypermethylated status.

This was confirmed by the observation that important signaling pathways such as MAPK, ErbB, TGF-beta, and insulin signaling pathways were found more frequently mutated in proximal than in distal carcinomas (supplementary Table S10, available at Annals of Oncology online). As supportive evidence, we found a similar mutation bias in the PETACC3 dataset for oncogenes, such as BRAF, KRAS, and PIK3Ca (supplementary Table S1, available at Annals of Oncology online).

We explored the association of tumor side with relapse-free survival (RFS) and survival after relapse (SAR) in the PETACC3 cohort. Surprisingly, stage II proximal carcinomas relapsed significantly less frequently than those in the distal colon (supplementary Figure S7, available at Annals of Oncology online). However, this appeared to be entirely due to the MSI population (mostly proximal), as this was no longer found when only MSS carcinomas were considered. For stage III patients, no effect of side was found on RFS (supplementary Figure S8, available at Annals of Oncology online). Multivariable analysis confirmed that side is not an independent prognostic factor for RFS (supplementary Table S11, available at Annals of Oncology online).

In contrast, when stage III patients with a proximal carcinoma became metastatic, they had a significantly worse survival than those with a metastatic distal carcinoma [HR 1.97, 95% CI (1.6–2.3), $P < 0.001$; supplementary Figure S7, available at Annals of Oncology online]. Multivariable analysis showed that this effect was independent of MSI and KRAS or BRAF mutation status [HR 1.7, 95% CI (1.3–2.4), $P < 0.001$; supplementary Table S11, available at Annals of Oncology online]. The BRAF signature score, which is higher in proximal carcinomas and itself highly prognostic for SAR [7], outcompeted side in a multivariable model (data not shown), although in the non-BRAF mutant-like subset side was still a significant factor (supplementary Figure S8, available at Annals of Oncology online).

In the smaller stage II proximal carcinoma cohort, we also observed a trend toward poorer outcome. This was confirmed in an independent untreated population (supplementary Figure S8, available at Annals of Oncology online).

In view of our finding that, in distal tumors, the frequency of amplification of ErbB family members is higher and the activation of EGFR signaling stronger, we explored if EGFR inhibitor efficacy is affected by tumor side. To this end, we studied 435 metastatic chemorefractory patients (126 or 29% proximal and 309 or 71% distal), of whom 207 were KRAS and BRAF wild-type (WT2) and had been treated with cetuximab combined with chemotherapy [19].

Overall, in univariable models, patients with a distal carcinoma showed better progression-free survival (PFS; 21 weeks (95% CI 19–24 weeks]) than those with a proximal carcinoma [13 weeks (95% CI 11–17 weeks); $P < 0.001$; supplementary Figure S9, available at Annals of Oncology online]. This was largely due to patients with a WT2 carcinoma, of whom the median PFS was 18 weeks in case of a proximal carcinoma (95% CI 11–31 weeks) but 30 weeks in case of a distal carcinoma (95% CI 26–34 weeks, $P = 0.02$). In contrast, KRAS or BRAF-mutated carcinomas did not show any difference in outcome according to side (data not shown).

**Discussion**

It is now clear that CRC is a molecularly heterogeneous disease [3–6], and that this heterogeneity should be used to stratify patients for optimal response to current and novel therapeutic strategies. We confirm the emerging notion that a significant part of this heterogeneity is captured by the anatomic location of the tumor. However, we were not able to confirm that these differences are significant when considering all samples or focusing exclusively on microsatellite stable (MSS), BRAF, and KRAS wild-type patients.
changes occur gradually along the bowel, as previously hypothesized [10].

We found differences in gene expression between the proximal and distal normal colon, which mostly overlapped with those found by LaPointe et al. [20], but which did not emerge as significant in the differences between proximal and distal carcinomas.

We confirm that proximal tumors are more often MSI and hypermutated, which is at least in part due to their deficient DNA mismatch repair status. However, in both the PETACC3 and TCGA series, even non-hypermutant proximal MSS carcinomas harbor more potentially deleterious mutations, including mutations of KRAS, BRAF, and PIK3Ca. We observed a higher frequency of BRAF-mutated, BRAF score, and serrated signature expressing proximal carcinomas, as was also found in mouse models recapitulating human BRAFV600E mutated serrated lesions with an MSI phenotype [21]. Proximal carcinomas, often characterized mucinous, densely infiltrated with tumor-infiltrating lymphocytes, and with activated MAPK signaling, might develop from precursor lesions driven by pathways which are associated with side-specific cellular characteristics, such as tolerance to DNA repair defects and to oncogenic stress. In addition, environmental factors like bacterial toxins or mutagenic CYP450 metabolites, which increase the mutation rate, may contribute to the specific characteristics of these cancers [11].

In contrast, distal carcinomas characteristically harbor numerous large chromosomal alterations (notably gain of 20q and loss of 18q), for which the responsible mechanisms are not fully understood. Loss of 18q [22] as well as activation of EGF signaling, which induce the expression of AURKA [23], might be implicated. We found HER1 and HER2, directly druggable targets, amplified in 12% of distal carcinomas (9% of which wild-type for KRAS and BRAF) and gene expression evidence of activation of the EGFR pathway largely restricted to the distal colon. The observation that, in the adenomatous polyposis coli mouse model, the disruption of the pan-ErbB-negative regulator LRIG1 predominantly induces distal neoplasms [24] supports the hypothesis of an important contribution of EGF signaling to distal colon carcinogenesis.

These differences in mutation rate and genomic instability between the two colon sides are striking and need to be better understood both in terms of their bearing on prognosis as well as response to DNA repair targeting chemotherapies. Multivariable analyses, containing all major known risk factors including BRAF and KRAS mutations, showed that side is an independent prognostic factor for SAR. Furthermore, BRAF mutant or BRAF-

Figure 2. Distribution of key variables ranked by BRAF score level in PETACC3 (N = 589) (A) and TCGA colon adenocarcinoma collection (N = 314) (B). From top to bottom panel, the plots depict: (1) ranking of the BRAF score, (2) serrated adenoma score, and (3) MSI-like score. These scores showed a strong association in both datasets. (4) Copy number variations of chromosomal locus of 43 receptor tyrosine kinases (RTKs) (PETACC3 dataset). The number of RTKs per patients showing gain (one copy) and amplification (more than two copies) is plotted. Patients showing amplification of HER1 and/or HER2 are also marked. A smooth curve was fitted by Loess (local polynomial regression fitting) using smoother span of 60%. In TCGA, we plotted the number of non-silent mutations observed per patient. A smooth curve was also fitted. (5) The median expression of the genes included in the EGFR signature set from Kobayashi et al. (UP and DW) (supplementary Materials and Methods, available at Annals of Oncology online) is reported as heatmap. (6) Distribution of tumors by side (red—proximal and blue—distal), MSI (red—MSI-H and blue—MSI), and BRAF status (red—BRAF mutant and blue—BRAF wild type).
like distal carcinomas have poorer SAR and RFS [7]. We hypothesize that metastases of proximal colon carcinomas have an increased mutation rate and higher cellular plasticity, potentially exacerbated by the effects of chemotherapy, with as a potential consequence a deleterious effect of (neo)adjuvant therapy. The combination of hypermethylation and a hypermutant state may induce, in metastases of proximal carcinomas, resistance to the chemotherapy, with as a potential consequence a deleterious effect of (neo)adjuvant therapy. The exacerbation of proximal colon carcinomas might require completely different drug regimens.

Our observations of an active EGFR signaling in distal carcinomas also suggest that those tumors benefit significantly more from anti-EGFR agents than proximal carcinomas, which was supported by our results obtained from a single-arm study. This finding also emerged recently from the NCIC-CTG-CO.17 reanalysis of cetuximab monotherapy versus best supportive care and emphasizes that benefit is restricted to proximal carcinomas [25].

In summary, the molecular and clinical characteristics of proximal and distal colon carcinomas are significantly different (as is summarized in Figures 2 and 3) and show to go beyond the simple MSI–MSS grouping. It remains to be seen if the findings hold also in advanced diseases, under-represented in our study. Tumor location is yet another simplistic subdivision of CRC, but it does go along with significant and characteristic molecular heterogeneity based on differences in biology, which is potentially highly relevant for therapeutic decision-making.

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references

Figure 3. Summary of the main biological and clinical findings. SAR, survival after relapse; MSAR, median survival after relapse; y, year.

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Fourfold increased detection of Lynch syndrome by raising age limit for tumour genetic testing from 50 to 70 years is cost-effective

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**Background:** Recognising colorectal cancer (CRC) patients with Lynch syndrome (LS) can increase life expectancy of these patients and their close relatives. To improve identification of this under-diagnosed disease, experts suggested raising the age limit for CRC tumour genetic testing from 50 to 70 years. The present study evaluates the efficacy and cost-effectiveness of this strategy.

**Methods:** Probabilistic efficacy and cost-effectiveness analyses were carried out comparing tumour genetic testing of CRC diagnosed at age 70 or below (experimental strategy) versus CRC diagnosed at age 50 or below (current practice). The proportions of LS patients identified and cost-effectiveness including cascade screening of relatives, were calculated by decision analytic models based on real-life data.

**Results:** Using the experimental strategy, four times more LS patients can be identified among CRC patients when compared with current practice. Both the costs to detect one LS patient (€9437/carrier versus €4837/carrier), and the number needed to test for detecting one LS patient (42 versus 19) doubled. When family cascade screening was included, the number needed to test increased to 62. The cost-effectiveness of this strategy compared with current practice was assessed using decision analytic models based on real-life data.

**Conclusions:** Raising the age limit for CRC tumour genetic testing from 50 to 70 years would increase the detection of Lynch syndrome patients, thereby increasing life expectancy and improving cost-effectiveness.