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Genetic Analysis Using a Gene Panel in 87 Caucasian Patients With Colorectal Cancer: Own Results and Review of Literature

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Abstract. *Background/Aim: Colorectal cancer (CRC) is the third most common cancer worldwide. The prognosis between left- and right-sided CRC differs, partly due to baseline differences as vascular supply. The purpose of the present study was to investigate whether there are genetic differences between left- and right-sided CRC. Patients and Methods: Eighty-seven patients with CRC (mean age: 61 years) were retrospectively included in the study. Blood samples were used for genetic analysis, by applying the sequencing research panel Ion AmpliSeq Colon and Lung Cancer Research Panel V2. Statistical analyses included Chi-square tests, Kaplan-Meier survival curves, and univariate/multivariate Cox-regression analyses. Results: By testing the sequence of 22 genes included in the panel, a significant difference was detected between left- and right-sided CRC regarding the expression of BRAF and DDR2 genes, with mutations occurring more often in the right-sided CRC. In the multivariate setting, left-sided CRC only turned out as a significant positive prognostic parameter regarding progression-free survival, irrespective of the type of chemotherapy or BRAF and NRAS mutations. Conclusion: Tumour location was the only parameter proven to be an independent prognostic factor for CRC in the present study.*

Colorectal cancer (CRC) is responsible for over 600,000 deaths per year and constitutes the third most common cancer worldwide (1). Risk factors for development of CRC include inflammatory bowel disease, obesity, unhealthy diet, smoking and alcohol consumption as well as family history of CRC (2-7). Several favourable prognostic and predictive markers have been discovered over the years, including high-level microsatellite instability, tumour-infiltrating lymphocytes (especially CD45R0- and CD3-positive T cells) and the presence or absence of *KRAS* mutations (8-10).

Generally, the left-sided colon and rectal cancer have a significantly better outcome than the right-sided colon cancer. This is partly due to baseline differences between left- and right-sided colon, such as embryological beginnings, the vascular supply, the length of colonic crypts and the frequency of mutations within the tumour itself (11-13).

In the metastatic setting, patients with resectable metastases may undergo surgery, whilst others should be offered palliative chemotherapy (9). Significant treatment advances have been made over the years, with substances such as bevacizumab, which targets the vascular endothelial growth factor (VEGF), and cetuximab, which inhibits the activity of the epidermal growth factor receptor (EGFR) (14). However, cetuximab is only allowed in patients with no mutations in the *RAS* gene (9).

In the past, several gene mutations including *NRAS, BRAF*, and *KRAS* have been discovered as being of prognostic significance in CRC (15-17). These three genes encode kinases of the RAS-RAF-MAPK pathway and are downstream effectors of the *EGFR* signalling pathway (18). *RAS* mutations convert the proto-oncogene to an activated oncogene, resulting in the continuous activation of the downstream MAPK-pathway (19).

In the present study, 22 genes frequently mutated in CRC were analyzed so as to associate their state with the tumour location, and their potential impact as prognostic parameters was examined. Furthermore, a literature review was performed on differences between left- and right-sided colon cancer regarding patient outcome and molecular-pathological characteristics.

Patients and Methods

Patients. Eighty-seven Caucasian patients with metastatic CRC treated between 2010 and 2017 at the Division of Clinical Oncology, Internal Medicine, Medical University of Graz, in Austria, were retrospectively included in the study. Demographic and pathological data, treatment-related, and follow-up information were derived from

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fever charts, pathology reports, and medical records. The median follow-up from start of palliative chemotherapy was 8 months [interquartile range (IQR)=3.5-12.5]. The mean age of all patients was 61.0 years (range=25-85) and 50/87 (57.5%) patients were male. Patients not receiving palliative chemotherapy, as well as patients with insufficient clinical data were excluded from the study.

Next generation sequencing (NGS) analysis. NGS libraries were prepared using the AmpliSeq library kit 2.0 (Thermo Fisher Scientific, Waltham Massachusetts, USA) and the Ion AmpliSeq Colon and Lung Cancer Research Panel V2 (Catalogue number: CP1004) primer pool covering hotspot mutations in 22 genes implicated in cancer. Patients' blood samples were analysed following the manufacturers' instructions. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo Fisher Scientific, Waltham Massachusetts, USA) to a length of 200 base pairs. Initial data analysis was done using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, https://github.com/iontorrent/). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. Called variants were annotated using open source software ANNOVAR (20) and SnpEff (21). All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (http://www.broadinstitute.org/igv/) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis.

Statistical analysis. Statistical analyses were performed using the SPSS Version 23.0. Progression-free survival (PFS) was calculated from the start of palliative chemotherapy to the date of disease progression. Chi-squared tests were performed to investigate differences between groups. Kaplan-Meier survivorship curves were applied to estimate differences between groups regarding PFS. Univariate and multivariate Cox regression models were used to assess hazard ratios (HRs) and 95% confidence intervals (95% CIs) for PFS.

Results

Twenty-four tumours were located on the right side (27.6%; from the caecum to the left colonic flexure) and 63 on the left side (72.4%; from the left colonic flexure to the distal rectum), from which 31 cases also included rectal cancers (Table I).

Sixty patients (69%) were already presented with metastases at the time of diagnosis. The remaining 27 patients (31.0%) developed metastatic disease after 11 months on average, following the diagnosis of CRC. As palliative treatment, 33 patients had only received chemotherapy (37.9%), 20 patients had been treated with anti-EGFR either alone or in combination with chemotherapy (23.0%) and 34 patients had been administered anti-VEGF alone or in combination with conventional chemotherapy (39.1%).

The mutation rate of the 22 genes is shown in Table II. Using Chi-squared test, we found that mutations in genes *BRAF* (p=0.026) and *DDR2* (p=0.020) were significantly more common in tumours located on the right side as compared to cancers emerging from the left side. The frequency of mutations in other genes, such as *KRAS, SMAD4, PIK3CA, NRAS, TP53, FBBX7, MET, FGFR3, MAP2K1, ERBB2, AKT1, PTEN* and *FGFR1* were not significantly different between left- and right-sided cancers. *CTNNB1, ALK, SKT11, EGFR, ERBB4, FGFR2* and *NOTCH1* were not mutated in any of the samples (Table III).

Table I. *Descriptive analysis of the study cohort*.

Characteristics	N (%)
Gender	
Male	50 (57.5%)
Female	37 (32.5%)
Tumour location	
Right-sided	24 (27.6%)
Left-sided	63 (72.4%)
Metastasis at presentation	
No	27 (31.0%)
Yes	60 (69.0%)
Chemotherapy	
CTX	33 (37.9%)
CTX + anti-EGFR	20 (23.0%)
CTX + anti-VEGF	34 (39.1%)

CTX, Chemotherapy, EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

In the univariate analysis, presence of mutations in NRAS (log-rank p=0.034) and BRAF (log-rank p=0.01) genes was significantly associated with a poorer PFS (Figures 1 and 2). No differences in PFS were found for *KRAS, SMAD4, TP53, DDR2, PIK3CA, FBBX7, MET, FGFR3, MAP2K1, ERBB2, PTEN, AKT1* and *FGFR1*. As *CTNNB1, ALK, SKT11, EGFR, ERBB4, FGFR2* and *NOTCH1* were wild type (WT) in all samples analysed, no Kaplan-Meier survival curves were calculated for these genes.

In the multivariate analysis, tumours located on the left side (HR=0.400, 95%CI=0.166-0.969, p=0.042) were significantly associated with a better PFS, irrespective of the *BRAF* and *NRAS* mutation status or type of chemotherapy (Table IV).

Discussion

In the present study, testing 22 genes by next generation sequencing revealed a significant difference between left- and right-sided colorectal cancer regarding the mutations in the *BRAF* and the *DDR2* gene, with both mutations occurring more often in the right-sided tumours. In the univariate analysis regarding the PFS of CRC patients, mutations in *NRAS* and *BRAF* genes were significantly associated with a poorer outcome. In the multivariate setting, however, only left-sided colon cancer was associated with a better PFS, irrespective of *BRAF* and *NRAS* mutation status or type of chemotherapy.

Table II. *Mutation rates of the genes included in the Ion AmpliSeq Colon and Lung Cancer Research Panel V2, in the total study population.*

KRAS (64.4%)	*EGFR* (0.0%)	*BRAF* (6.9%)	*PIK3CA* (13.8%)
ERBB2 (1.1%)	*PTEN* (1.1%)	*NRAS* (1.1%)	*STK11* (0.0%)
MAP2K1 (3.4%)	*ALK* (0.0%)	*DDR2* (2.3%)	*CTNNB1* (0.0%)
MET (4.6%)	*TP53* (44.8%)	*SMAD4* (8.0%)	*FBX7* (5.7%)
FGFR3 (1.1%)	*NOTCH1* (0.0%)	*ERBB4* (0.0%)	*FGFR1* (1.1%)
AKT1 (2.3%)	*FGFR2* (0.0%)		

Table III. *Mutation status depending on the tumour location (right vs. left side).*

		Right side CRC, n (%)	Left side CRC, n (%)	*p*-Value
NRAS	WT	23 (26.7)	63 (73.3)	0.103
	Mutation	1 (100.0)	0 (0.0)	
KRAS	WT	6 (19.4)	25 (80.6)	0.201
	Mutation	18 (32.1)	38 (67.9)	
BRAF	WT	20 (24.7)	61 (75.3)	**0.026**
	Mutation	4 (66.7)	2 (33.3)	
SMAD4	WT	24 (30.0)	56 (70.0)	0.089
	Mutation	0 (0.0)	7 (100.0)	
TP53	WT	14 (29.2)	34 (70.8)	0.714
	Mutation	10 (25.6)	29 (74.4)	
DDR2	WT	22 (25.9)	63 (74.1)	**0.020**
	Mutation	2 (100.0)	0 (0.0)	
PIK3CA	WT	22 (29.3)	53 (70.7)	0.362
	Mutation	2 (16.7)	10 (83.3)	
FBBX7	WT	23 (28.0)	59 (72.0)	0.696
	Mutation	1 (20.0)	4 (80.0)	
MET	WT	22 (26.5)	61 (73.5)	0.304
	Mutation	2 (50.0)	2 (50.0)	
CTNNB1	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
FGFR3	WT	24 (27.9)	62 (72.1)	0.535
	Mutation	0 (0.0)	1 (100.)	
MAP2K1	WT	23 (27.4)	61 (72.6)	0.821
	Mutation	1 (33.3)	2 (66.7)	
ERBB2	WT	23 (26.7)	63 (73.3)	0.103
	Mutation	1 (100.0)	0 (0.0)	
PTEN	WT	23 (26.7)	63 (73.3)	0.103
	Mutation	1 (100.0)	0 (0.0)	
AKT	WT	24 (28.2)	61 (71.8)	0.377
	Mutation	0 (0.0)	2 (100.0)	
ALK	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
STK11	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
EGFR	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
ERBB4	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
FGFR1	WT	24 (27.9)	62 (72.1)	0.535
	Mutation	0 (0.0)	1 (100.0)	
FGFR2	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	
NOTCH1	WT	24 (27.6)	63 (72.4)	n/a
	Mutation	0 (0.0)	0 (0.0)	

WT, Wild type; n/a, not applicable. Statistically significant *p*-values are shown in bold.

Left- and right-sided colon show different features, from the histogenetic origin – the right-sided colon arises from the midgut, whilst the left-sided colon comes from the hindgut – to the differential expression of genes (13). Moreover, distinct molecular pathways are responsible for the development of left- and right-sided tumours. Right-sided colon cancer rather presents in cases where there is microsatellite instability (MSI-H), a CpG island methylator phenotype (CIMP), as well as mutations in *KRAS, PIK3CA*, and *BRAF* genes (22-24). In the present study, only *BRAF* mutations occurred more frequently in the right-sided tumours, together with the *DDR2* mutations. In a large study by Slattery *et al*., 320 genes were identified being differentially expressed between colon cancers located on the right and left side at a significance level of 0.05 and 116 genes at a significance level of 0.01 (25). The study group also discovered a significant difference in gene expression levels depending on the extend of CIMP and MSI, and, moreover, revealed that the deregulation of mucin genes plays a crucial role in MSI-high tumours (25). Related to this, a previous study by Tanaka *et al*. discovered that the methylation status of *hMLH1* gene, which is a main cause of MSI and is also associated with hereditary nonpolyposis colorectal cancer (HNPCC), is less frequently in left-sided colon cancer, together with a less frequent occurrence of CIMP positive state (26).

Notably, in a large study of 1,443 colon cancer samples, it was shown that there is no specific physical border at the left colonic flexure regarding the different molecular features displayed in CRC. Instead, mutation profiles change constantly from the proximal to the distal colon (*i.e*. from the cecum to the colon ascendens, to the right colonic flexure, to the colon transversum, to the left colonic flexure, to the colon descendens, to the – sigmoid, and finally to the – rectum) (27). However, as in the study by Tanaka *et al*. (26), high levels of MSI were more commonly seen in the right-sided colon cancer.

Besides the status of either MSI or CIMP, chromosomal instability (CIN) constitutes the third main type of (epigenetic) instability in CRC (28). CIN develops predominantly in sporadic tumours going through the adenoma-carcinoma sequence (29). Related to this, the most frequent gene mutations detected in our study were *KRAS* (64.4%, followed by *TP53* (44.8%), *PIK3CA* (13.8%) and *SMAD4* (8.0%), all associated with CIN (30). Our results are in line with the study by Wang *et al*. in which tumour specimens from 648 CRC patients were analysed using an amplicon-based targeted next generation sequencing (NGS) assay (24).

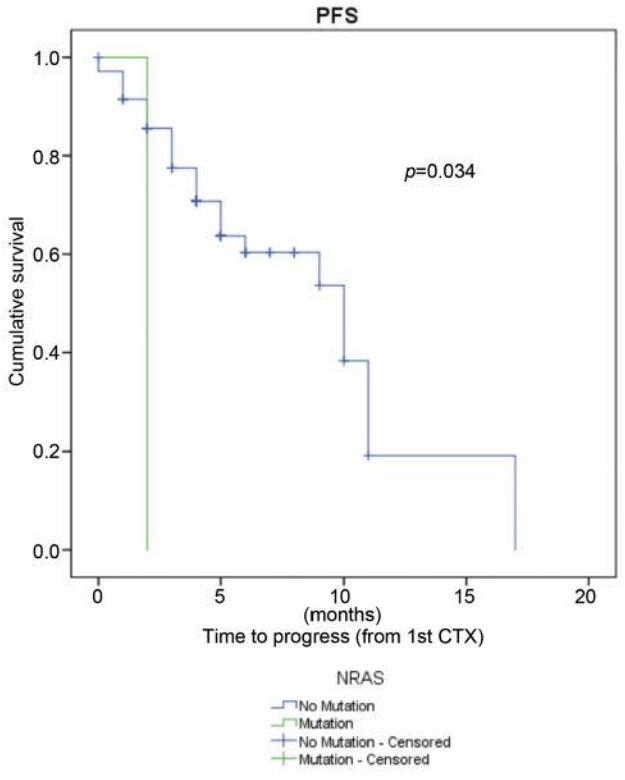

Figure 1. *Kaplan–Meier survival curve depicting improved survival for patients without an NRAS mutation in the univariate analysis. PFS, Progression-free survival; CTX, chemotherapy.*

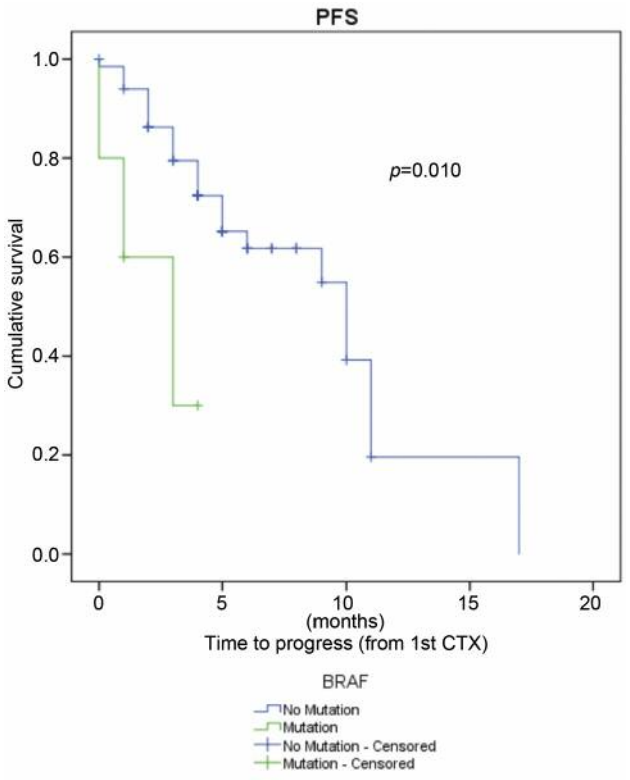

Figure 2. *Kaplan–Meier survival curve showing the improved survival for patients without BRAF mutations in the univariate setting. PFS, Progression-free survival; CTX, chemotherapy.*

Considering the prognostic significance of gene mutations in colon and rectal cancers, several studies have been performed in the past. For example, expression of DDR2 is associated with a poor patient outcome (31). In another study, *KRAS* mutations were not only associated with a poorer disease-free survival, but also with good response to FOLFOX-based chemotherapy (15). *BRAF* mutations, most commonly at codon 600, have been shown to predict poor outcome in CRC-patients (16). Similarly, NRAS mutations have been associated with a poorer overall survival in patients with CRC (17).

In the present study, mutations in *BRAF* and *NRAS* genes were significantly associated with a worse PFS in the univariate analysis. However, this association was not statistically significant in the multivariate setting, with the addition of tumour location, and the different types of therapy provided (*EGFR*-based, *VEGF*-based or conventional chemotherapy). In the study by Lee *et al*., mutations in the receptor tyrosine kinase (RTK)-RAS pathway (*e.g. BRAF, EGFR, KRAS, HRAS*) were associated with a significantly shorter recurrence-free survival, but only for left-sided tumours (32). On the other hand, *BRAF* mutations did not have a negative prognostic role in that study (27), despite being confirmed as a negative prognostic parameter in other analyses (32-34). Likewise in our study, *BRAF* mutations were associated with a poor patient outcome in the univariate setting. Additionally, in a randomised phase III NCCTG (Alliance) N0147 adjuvant chemotherapy trial that included 2,720 stage III colon cancers, patients harbouring *KRAS* or -mutations had a significantly reduced 5-year PFS (35). Interestingly, according to the PETACC3 adjuvant chemotherapy trial, right-sided stage II colon cancers developed relapses less often compared to left-sided cancers, whilst this difference was not evident in stage III tumours (13). Taken together, there are significant differences in mutation status between left- and right- sided colon cancer. However, results strongly depend on analytical tools used, selection of tumour types, and patient ethnicity included.

In the light of precision medicine era, the identification of markers predicting the risk for individual patients to develop CRC has become important. Depending on ethnicity, genetic alterations may significantly differ. For example, in a study by Yueh *et al*., a specific genotype of the matrix metalloproteinase 7 (MMP-7) gene, MMP-7 A-181G, was associated with a slightly decreased susceptibility to develop CRC in comparison to the wild type C-153T in Taiwanese patients (36). In a related study, the single nucleotide polymorphism (SNP309) G allele

Table IV. *Multivariate Cox-regression analysis for progression-free survival (PFS).*

	HR	Lower	Upper	*p*-Value
BRAF				
WT	1			0.101
Mutation	3.152	0.798	12.446	
NRAS				
WT	1			0.068
Mutation	8.093	0.856	76.485	
CTX				
CTX only	1			0.165
Anti-EGFR +CTX	1.590	0.610	4.145	
Anti-VEGF +CTX	0.492	0.181	1.340	
Location				
Right	1			0.042
Left	0.400	0.166	0.969	

HR, Hazard ratio; WT, wild type; CTX, chemotherapy; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

in the murine double minute 2 (MDM2) gene significantly increased the susceptibility to CRC in comparison to the wild type T allele (37). In another study on Taiwanese patients, the methylenetetrahydrofolate (MTHFR) rs1801133 T allele conferred to a lower CRC susceptibility compared to the wild type C allele (38).

Moreover, according to Shamoun *et al*., alterations of interleukin-32 (IL-32), an intracellular pluripotent cytokine, correlated with dissemination of CRC in Swedish patients (39). In Caucasian patients, Cullin 1 (CUL-1) and CUL-2 proteins, which play a role in cell proliferation, migration, and invasion, were also shown to be involved in early carcinogenesis, as they were found in adenomatous polyps of the large bowel (39). Considering that specific genetic alterations are prevalent depending on ethnicity, further in-depth research comparing biomarkers between different countries would be of great value.

One limitation of the present study is the relatively small and heterogeneous patient cohort, including patients with primary as well as with metachronous metastasis. Additionally, the patients in the study cohort had received different treatments, partially based on their mutation profile (*i.e. KRAS, NRAS, BRAF* mutation status) and their clinical presentation. Despite the limitations, a significant difference between left- and right-sided colon cancer regarding the presence of *DDR2* and *BRAF* mutations was shown. However, tumour sidedness was the only powerful prognostic factor in CRC, regardless of the *NRAS* or *BRAF* mutation status or the type of chemotherapy provided.

In synopsis, we and others have found significant differences between left- and right-sided colon cancer with regards to molecular-pathological profiles, However, not all of these parameters are reproducible in every single study, due to the genetic background of different populations and the different sensitivity of the various methods used to detect and interpret genetic alterations (40). Further larger studies on the identification of specific CRC biomarkers of prognostic value would enable tailored treatment for CRC patients.

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