Supplementary material

A higher ctDNA fraction decreases survival in regorafenib-treated metastatic colorectal cancer patients. Results from the regorafenib's liquid biopsy (RELAIS) translational biomarker phase II pilot study.

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1) Supplementary tables:

Table S1. Genes enriched using the SureSelect Custom Panel

| Symbol	Description*	Symbol	Description*
ANGPT1	Angiopoietin 1	**KRAS**	**KRAS Proto-Oncogene, GTPase**
ANGPT2	Angiopoietin 2	*MAPK1*	Mitogen-Activated Protein Kinase 1
ANGPT4	Angiopoietin 4	*MAPK11*	Mitogen-Activated Protein Kinase 11
APC	**Adenomatosis Polyposis Coli Tumor Suppressor**	*MET*	MET Proto-Oncogene, Receptor Tyrosine Kinase
BRAF	**B-Raf Proto-Oncogene, Serine/Threonine Kinase**	*MMP9*	Matrix Metallopeptidase 9
CDC42	Cell Division Cycle 42	*MRAS*	Muscle RAS Oncogene Homolog
COL18A1	Collagen Type XVIII Alpha 1 Chain	*NFKB1*	Nuclear Factor Kappa B Subunit 1
CUL1	Cullin 1	*NFKB2*	Nuclear Factor Kappa B Subunit 2
CXCL8	C-X-C Motif Chemokine Ligand 8	***NRAS***	**NRAS Proto-Oncogene, GTPase**
CXCR1	C-X-C Motif Chemokine Receptor 1	*NRP1*	Neuropilin 1
CXCR2	C-X-C Motif Chemokine Receptor 2	*NRP2*	Neuropilin 2
CXCR4	C-X-C Motif Chemokine Receptor 4	*PDGFRA*	Platelet Derived Growth Factor Receptor Alpha
DDR2	Discoidin Domain Receptor Tyrosine Kinase 2	*PDGFRB*	Platelet Derived Growth Factor Receptor Beta
DLL4	Delta Like Canonical Notch Ligand 4	*PIGF*	Phosphatidylinositol Glycan Anchor Biosynthesis Class F
EPHA2	EPH Receptor A2	***PIK3CA***	**Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha**
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2	*PLG*	Plasminogen
FCGR2A	Fc Fragment Of IgG Receptor IIa	*PROK2*	Prokineticin 2
FGF2	Fibroblast Growth Factor 2	*PTEN*	Phosphatase And Tensin Homolog
FGFR1	Fibroblast Growth Factor Receptor 1	*PTGS2*	Prostaglandin-Endoperoxide Synthase 2
FGFR2	Fibroblast Growth Factor Receptor 2	*RAF1*	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
FGFR3	Fibroblast Growth Factor Receptor 3	*RBX1*	Ring-Box 1
FGFR4	Fibroblast Growth Factor Receptor 4	*REL*	REL Proto-Oncogene, NF-KB Subunit
FIGF	Vascular Endothelial Growth Factor D, FIGF	*RELA*	RELA Proto-Oncogene, NF-KB Subunit
FLT1	Fms Related Tyrosine Kinase 1	*RELB*	RELB Proto-Oncogene, NF-KB Subunit
FLT4	Fms Related Receptor Tyrosine Kinase 4	*RET*	Ret Proto-Oncogene
FRK	Fyn Related Src Family Tyrosine Kinase	*SKP1*	S-Phase Kinase Associated Protein 1
HGF	Hepatocyte Growth Factor	***SMAD4***	**SMAD Family Member 4**
HRAS	**HRAS Proto-Oncogene, GTPase**	*TEK*	TEK Receptor Tyrosine Kinase
IGF1	Insulin Like Growth Factor 1	*TIE1*	Tyrosine Kinase With Immunoglobulin Like And EGF Like Domains 1
IGF1R	Insulin Like Growth Factor 1 Receptor	*TNF*	Tumor Necrosis Factor
IL6	Interleukin 6	***TP53***	**Tumor Protein P53**
JAK2	Janus Kinase 2	*VEGFA*	Vascular Endothelial Growth Factor A
KDR	Kinase Insert Domain Receptor	*VEGFC*	Vascular Endothelial Growth Factor C
KIT	KIT Proto-Oncogene Receptor Tyrosine Kinase	*VWF*	Von Willebrand Factor

*according to GeneCards, most frequently mutated gene in CRC are highlighted in bold

Table S2. patients´ characteristics

Patient ID	Sex	Age	OS[1] [days]	PFS[2] [days]	Tumor	Localization	Metastases				RAS mutation in tussue	RAS mutation in plasma	Duration of therapy with regorafinib	Response to treatment[3]	ECOG
							Lung	Liver	LN	Other					
R1	m	49	1182	195	rectum	left-sided	yes	yes	no	1	*KRAS* G12C	*KRAS* G12C	195	SD	1
R2	m	75	1348	132	sigmoid colon	left-sided	yes	yes	no	no			132	SD	0
R3	w	66	1034	118	rectum	left-sided	yes	yes	no	no			118	SD	0
R4	m	66	1052	86	rectum	left-sided	yes	yes	yes	no	*KRAS* G13D	*KRAS* G13D	86	SD	0
R5	m	78	3555	449	sigmoid colon	left-sided	yes	yes	yes	no		*KRAS* G12A	449	SD	1
R6	m	59	1149	58	coecum	left-sided	no	yes	yes	yes	*KRAS* G12D	*KRAS* G12D	58	PD	0
R8	m	69	674	28	rectum	left-sided	yes	yes	yes	yes			28	EOT before CT	1
R9	w	38	728	34	sigmoid colon	left-sided	yes	yes	yes	yes	*KRAS* G13D	*KRAS* G13D	34	EOT before CT	1
R10	w	50	1305	82	sigmoid colon	left-sided	no	yes	yes	no	KRAS A146T	*KRAS* A146T	82	PD	0
R11	m	71	1284	36	sigmoid colon	left-sided	yes	no	yes	no		*KRAS* Q61H	36	PD	1
R13	w	70	1278	26	sigmoid colon	left-sided	no	yes	no	no			26	PD	1
R14	m	52	1378	99	ascending colon	right-sided	yes	yes	no	no	NRAS Q61R	*NRAS* Q61R	99	PD	0
R15	m	33	1306	94	appendix	right-sided	no	yes	yes	yes	*KRAS* G12D	*KRAS* G12D	94	PD	0
R16	m	68	1079	63		right-sided	no	yes	yes	yes			63	EOT before CT	0
R17	m	78	750	140	ascending colon	right-sided	no	yes	no	no		*KRAS* G12A	140	SD	0
R18	m	54	816	84	rectum	left-sided	yes	yes	yes	yes	*KRAS* G12V	*KRAS* G12V	84	SD	1
R19	m	70	255	33	rectum	left-sided	no	yes	yes	yes	*KRAS* G12D	*KRAS* G12D	33	EOT before CT	1
R20	m	46	1242	58	ascending colon	right-sided	yes	yes	yes	yes			58	PD	0
R21	m	52	912	85	rectum	left-sided	yes	yes	yes	no			85	PD	0
R22	m	72	451	30	rectum	left-sided	yes	yes	yes	yes			30	PD	1
R23	m	48	700	63	rectum	left-sided	yes	yes	yes	yes	*KRAS* G12V	*KRAS* G12V	63	PD	1
R24	w	69	336	43	ascending colon	right-sided	no	yes	yes	yes			43	PD	0
R25	w	57	2325	160	descending colon	left-sided	yes	yes	yes	no	*KRAS* G12D	*KRAS* G12D	160	PR	0
R26	m	56	458	26	ascending colon	right-sided	yes	yes	yes	yes			26	EOT before CT	1
R27	m	62	1600	79	ascending colon	right-sided	yes	yes	yes	yes	*KRAS* G12D		79	PD	1
R28	w	68	352	58	sigmoid colon	left-sided	yes	yes	yes	yes	*KRAS* G12D	*KRAS* G12D	58	PD	1
R29	m	58	720	17	ascending colon	right-sided	yes	yes	yes	yes			17	EOT before CT	1
R31	w	34	1499	113	sigmoid colon	left-sided	yes	yes	yes	yes			113	PD	0
R32	w	58	493	21	rectum	left-sided	yes	yes	yes	no			21	PD	0
R33	w	63	1305	112	sigmoid colon	left-sided	yes	yes	yes	no	*KRAS* G12S	*KRAS* G12S	112	PR	0

[1]OS, overall survival, [2]PFS, progression free survival, [3]PD, progressive disease; PR, partial response; SD, stable disease; EOT before CT, treatment was stopped due to side effects or progression before CT scan was scheduled; EOT before CT, clinical progress and/or death before a CT-scan

2) Supplementary Figures:
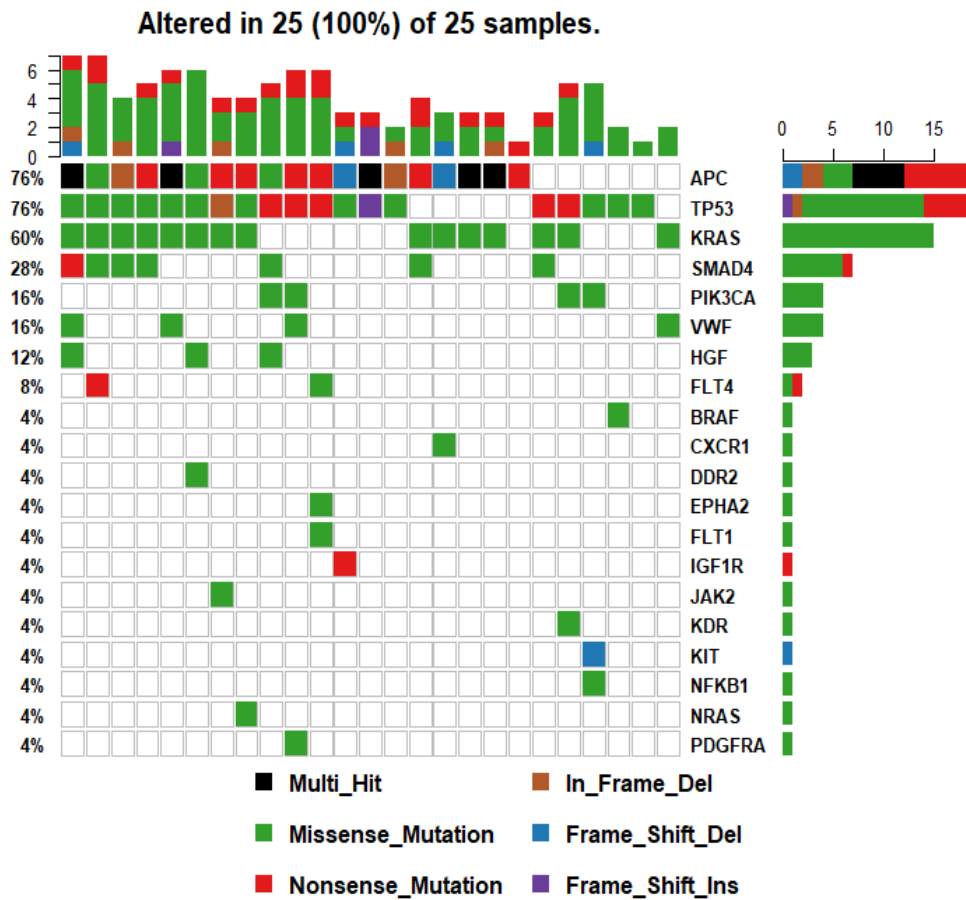
Figure S1. Oncoprint. *Heatmap showing the distribution of genomic alterations in the mCRC cohort. In 25/30 patients at least one mutation was detected. Shown is an overview of genomic alterations (legend) in particular genes (rows) affecting individual samples (columns).*
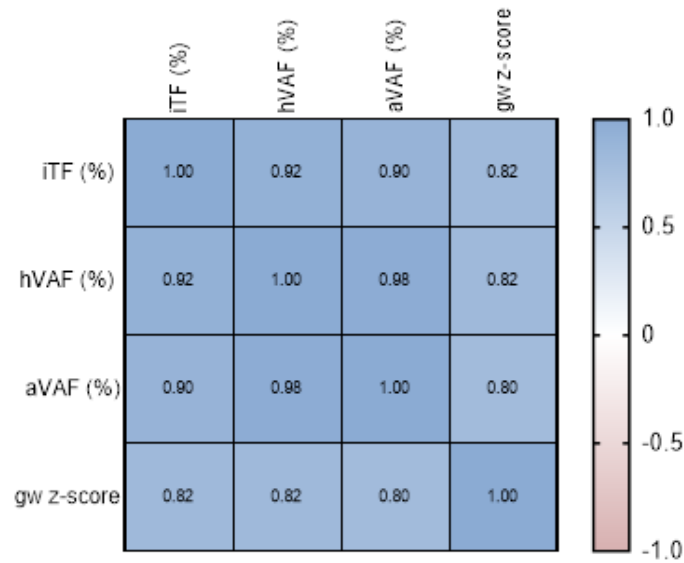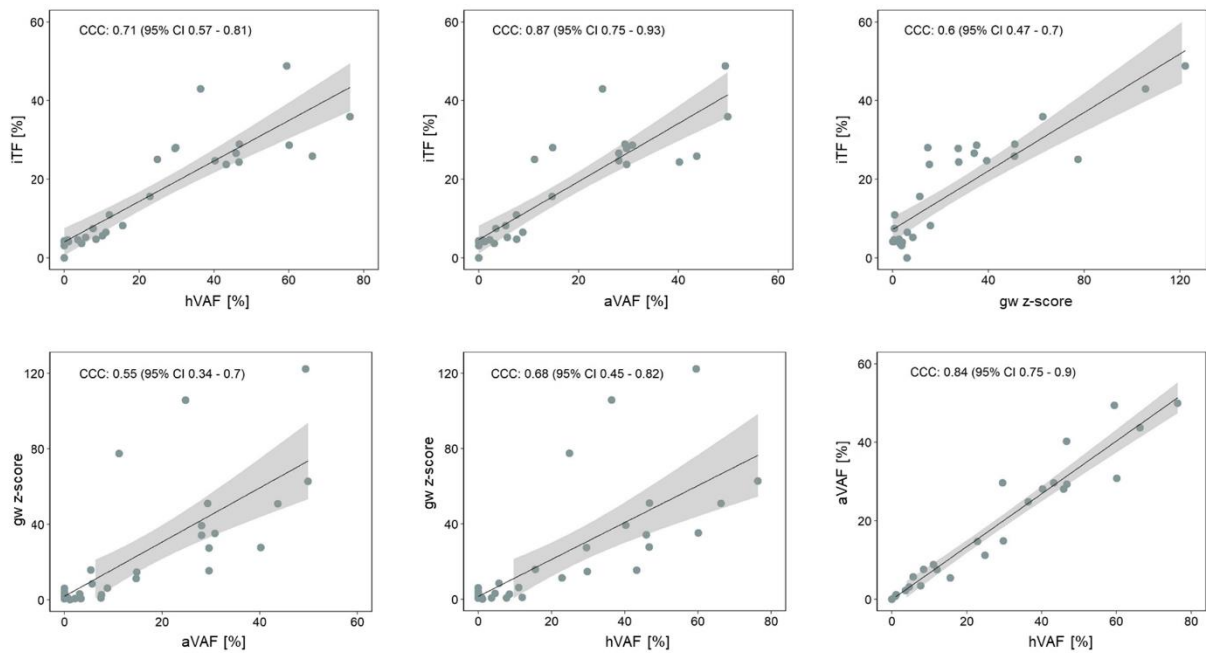
Figure S3. Correlation/Concordance of various proxies for ctDNA levels at baseline. *(A) Heatmap representing the Spearman correlation coefficients of ctDNA level assessed with ichorCNA-derived tumor fraction (iTF), the highest variant allele (hVAF) frequencies form the SureSelect panel, the average variant allele (aVAF) frequencies form all mutations identified with the SureSelect panel and the genomewide z-score (gw z-score) calculated from mFAST-SeqS. (B) Shown are linear regression of the Linear regression of various proxies including the Lin´s concordance coefficient (CCC).*
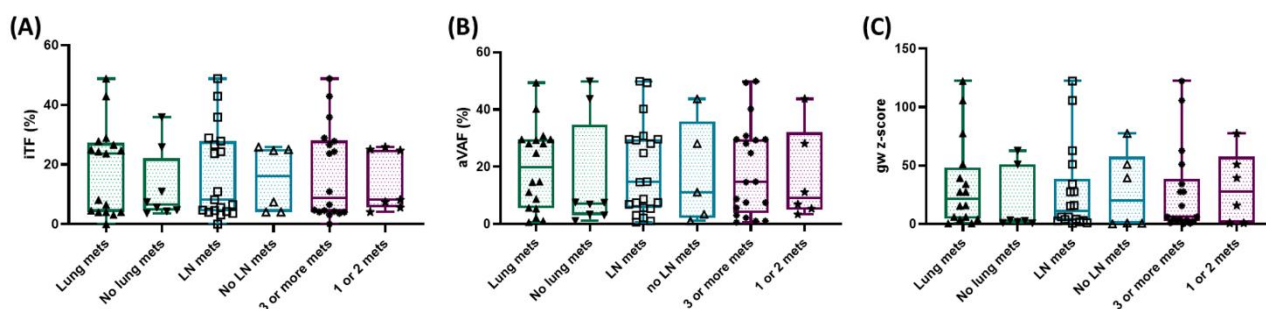
Figure S4. Association of baseline ctDNA levels and presence of metastases. *Shown are box plots of ctDNA levels represented as (A) ichorCNA-derived tumor fraction (iTF) (B) with the average variant allele (aVAF) frequencies and (C) gw zcore. No significant difference was observed between patient with or without lung or lymph node (LN) metastases or three or more metastatic sites*
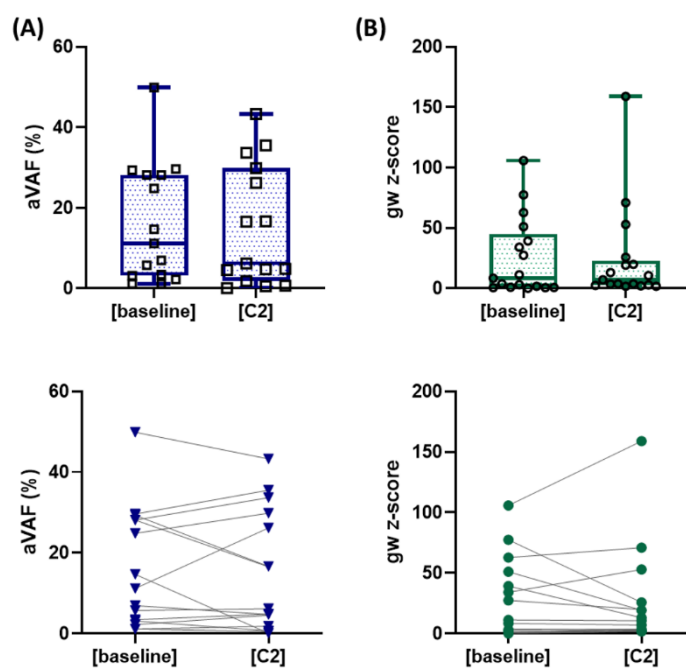
Figure S5. ctDNA levels at baseline and before treatment cycle 2. *Upper panel: Distribution of various proxies for ctDNA levels prior to treatment initiation (baseline) and treatment cycle 2 (C2). (A) gw z-score, genomewide z-score calculated from mFAST-SeqS and (B) aVAF, average variant allele frequency (VAF) identified with the SureSelect panel. Lower panel: Changing levels of ctDNA reflected as (A) gw z-score and (B) aVAF*