Supplementary Online Content

Montagut C, Argilés G, Ciardiello F, et al. Efficacy of Sym004 in patients with metastatic colorectal cancer with acquired resistance to anti-EGFR therapy and molecularly selected by circulating tumor DNA analyses: a phase 2 randomized clinical trial. Published online February 8, 2018. *JAMA Oncology*. doi:10.1001/jamaoncol.2017.5245

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

NIH-3T3 cells (ATCC) stably overexpressing WT *EGFR* or *EGFR* ECD mutations were generated using retroviral transduction. Full length human EGFR constructs were synthesized (by Genscript) and subcloned into the retroviral vector pQCXIP (Clontech). The presence of specific *EGFR* ECD mutations was confirmed by sequencing. A VSV-G pseudotyped retrovirus (Cell Biolabs) was produced using the Phoenix-AMPHO packaging cell line (ATCC), and filtered supernatants containing 8 µg/mL polybrene (Sigma) were used to infect NIH-3T3 cells. Cells were selected in 2 µg/mL puromycin (Thermo Fisher).
2-fold serial dilution of antibodies starting from 25 µg/mL was used to generate dose-response curves. Cells were cultured in the presence of antibodies in medium containing 2% FBS. After 96 hours of culture cell viability was determined using the WST-1 assay (Roche Diagnostics).
To assess antibody binding, A 4-fold serial dilution of unlabeled antibodies was used to generate dose-response curves. Transfected cells were washed and incubated with goat anti-human IgG (H+L)-Alexa Fluor® 647 (R&D Systems) at a 1:250 dilution for 30 minutes, before a fluorescence readout was measured using the iQue Screener platform (IntelliCyt).
For quantification of Total EGFR and pEGFR Levels by Simple Western, lysates were generated using Pierce RIPA buffer containing protease inhibitors (Thermo Scientific) and phosphatase inhibitors (Calbiochem). Samples for Simple Western analysis were diluted to 0.2 µg/µL in a master mix containing internal fluorescent standards and reducing agent, and were processed *per* standard protocol using a Sally Sue instrument (ProteinSimple). Antibodies against total EGFR (C74B9), pEGFR (Y1068), and Pan-Actin (all from Cell Signaling Technology) were diluted 1:50. CRC PDX tumor xenografts were derived from surgical specimens from cancer patients and were established and characterized at EPO-GmbH, Germany or Oncotest, Germany. After transplantation of 2×2 mm tumor fragments to NMRI-Foxn1nu mice, tumors were measured at least twice weekly. When tumors reached 50-250 mm3, preferably 80-200 mm3, animals were distributed into experimental groups with the aim of having comparable median and mean group tumor volumes of approximately 100-200 mm3, and treatment was initiated. The experiment was performed with 10 animals/group and three groups/model: vehicle control, Sym004, and cetuximab. Sym004 and cetuximab were administered at a dose of 30 mg/kg intraperitoneally (i.p.) twice weekly for 5 weeks (9-10 doses in total).

Analysis of the Patient Subgroup Excluded due to Medical Practice Inconsistent with the Standard Therapy of Patients with mCRC

For patients enrolled in the Sym004 Phase 2 study there was a notable disparity in OS between patients treated in Russia and those treated in other countries. Median OS for all treatments combined was 8.9 months for all patients excluding those in Russia (i.e., the EU and US only) vs. 13.9 months in Russia. The median duration of treatment (all arms) was nearly 4 times longer for patients from Russia (36 months) than for the EU and US patients (9.1 months). Also, 25% of the EU and US patients had EGFR ECD mutations vs. none of the patients from Russia. Because of these disparities, ad hoc analyses excluding patients enrolled by the Russian sites were done to remove this confounding country effect. The data obtained support the suggestion that the patients in Russia were less refractory to standard EGFR moAb / more sensitive to therapy in general and to treatment on the three arms of this protocol specifically.

PDX Models

CRC PDX tumor xenografts were derived from surgical specimens from cancer patients and were established and characterized at EPO-GmbH, Germany or Oncotest, Germany. After transplantation of 2×2 mm tumor fragments to NMRI-Foxn1nu mice, tumors were measured at least twice weekly. When tumors reached 50-250 mm3, preferably 80-200 mm3, animals were distributed into experimental groups with the aim of having comparable median and mean group tumor volumes of approximately 100-200 mm3, and treatment was initiated. The experiment was performed with 10 animals/group and three groups/model: vehicle control, Sym004, and cetuximab. Sym004 and cetuximab were administered at a dose of 30 mg/kg intraperitoneally (i.p.) twice weekly for 5 weeks (9-10 doses in total).

eFigure 1. Overview of the 70 genes included in the Guardant360 version 2.9 panel. Genes were sequenced in critical exon regions except for those highlighted in bold, where the full exon was sequenced.

POINT MUTATIONS/INDELS

AKT1	*ALK*	***APC***	***AR***	*ARAF*	***ARID1A***	*ATM*	***BRAF***	***BRCA1***	***BRCA2***
CCDN1	***CCND2***	***CCNE1***	*CDH1*	***CDK4***	***CDK6***	***CDKN2A***	***CDKN2B***	*CTNNB1*	***EGFR***
ERBB2	*ESR1*	*EZH2*	*FBXW7*	***FGFR1***	***FGFR2***	*FGFR3*	*GATA3*	*GNA11*	*GNAQ*
GNAS	*HNF1A*	***HRAS***	*IDH1*	*IDH2*	*JAK2*	*JAK3*	***KIT***	***KRAS***	*MAP2K1*
MAP2K2	***MET***	*MLH1*	*MPL*	***MYC***	***NF1***	*NFE2L2*	*NOTCH1*	*NPM1*	***NRAS***
NTRK1	***PDGFRA***	***PIK3CA***	***PTEN***	*PTPN11*	***RAF1***	***RB1***	*RET*	*RHEB*	*RHOA*
RIT1	*ROS1*	*SMAD4*	*SMO*	*SRC*	*STK11*	*TERT*	***TP53***	*TSC1*	*VHL*

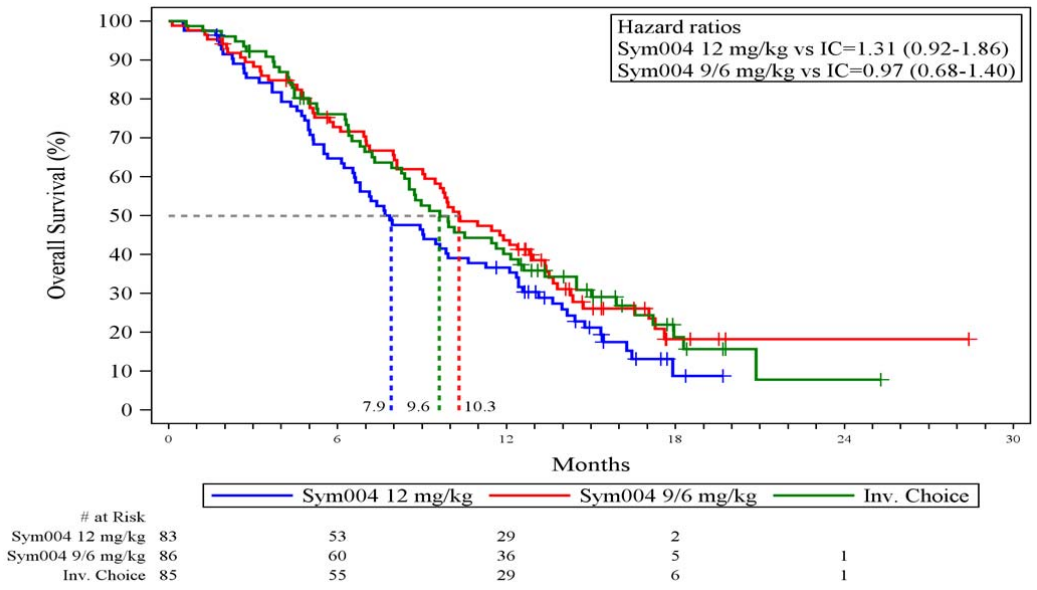
AMPLIFICATIONS

AR	*BRAF*	*CCNE1*	*CDK4*	*CDK6*	*EGFR*	*ERBB2*	*FGFR1*
FGFR2	*KIT*	*KRAS*	*MET*	*MYC*	*PDGFRA*	*PIK3CA*	*RAF1*

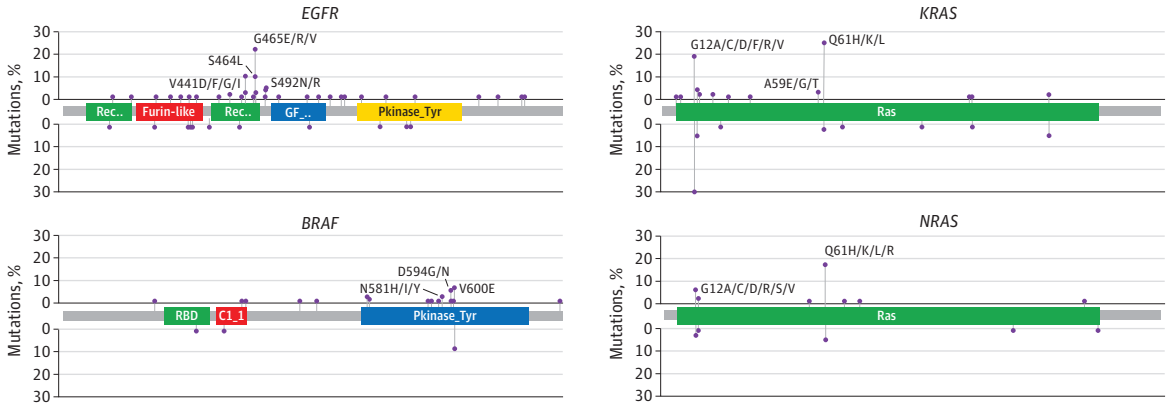
FUSIONS

ALK	*FGFR2*	*FGFR3*	*RET*	*ROS1*	*NTRK1*

eFigure 2. Kaplan-Meier survival estimates for Overall Survival in ITT population



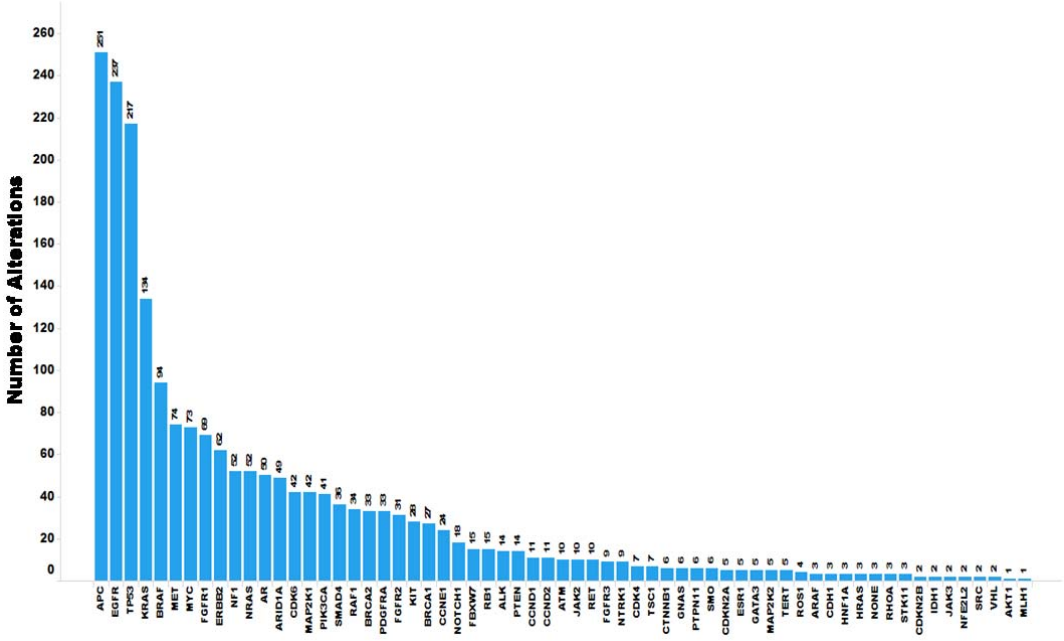
eFigure 3



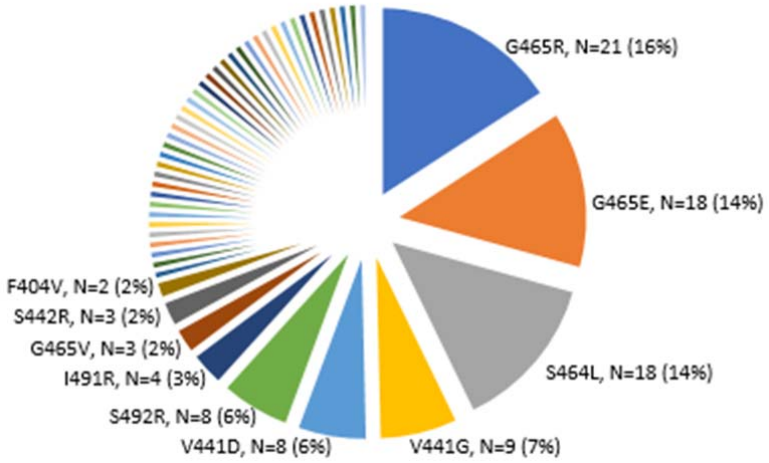
eFigure 3. Baseline Genotyping for Genomic Biomarker Analysis for Patients Included in the Sym004-05 Study

Lollipop plots of missense mutations identified in the EGFR, BRAF, KRAS, and NRAS genes in the present study (top half of each plot) compared with data obtained from The Cancer Genome Atlas (bottom half of each plot). Amino acid alterations detected at mutational hotspots are depicted for each gene.

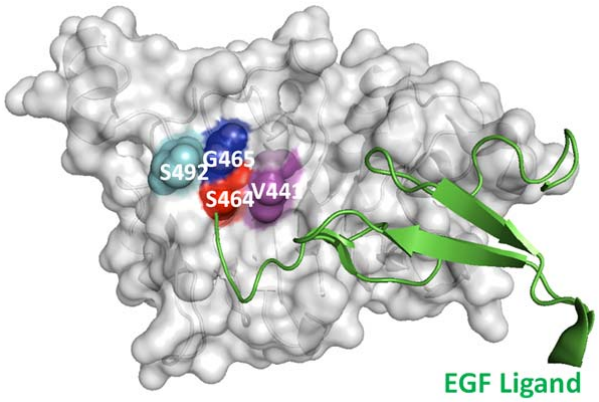
eFigure 4. Number of genomic alterations (single nucleotide variants, copy number variants, indels, and fusions) identified in circulating tumor DNA from patients (N=193), listed by gene.



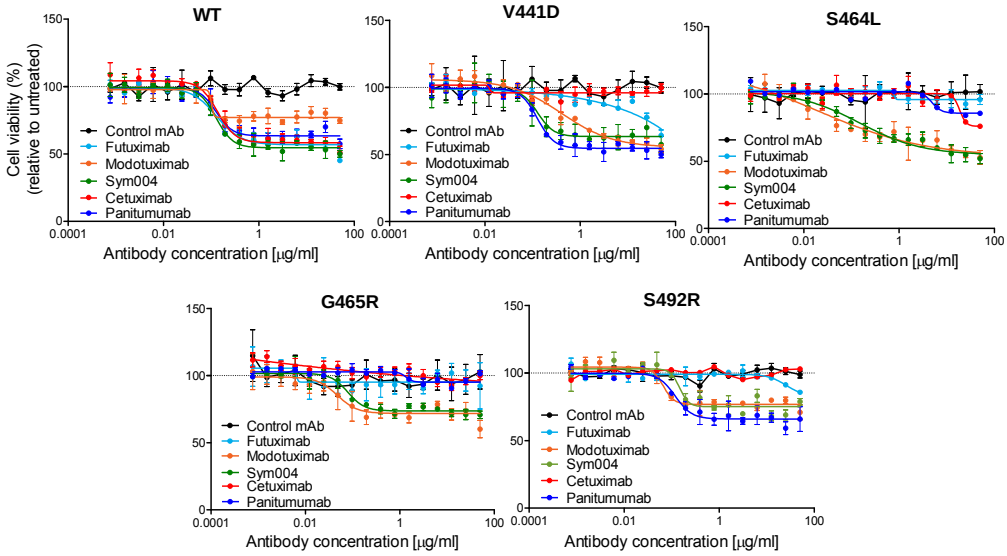
eFigure 5. Patient distribution and frequency of *EGFR* single nucleotide variant missense mutations. N=Number of patients with each mutation; %=Percentage of the total number of non-silent single nucleotide variant mutations in *EGFR* detected in the patients.



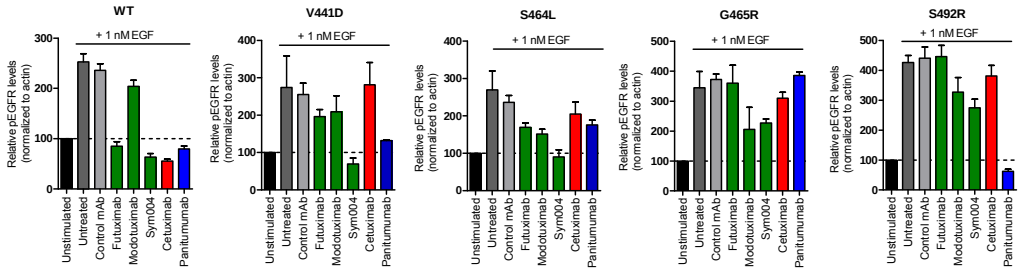
eFigure 6. Structural modeling of EGFR and mapping of *EGFR* ECD mutations identified in the patient cohort. The four amino acid positions that were most frequently mutated (G465, S464, V441, and S492) are highlighted.



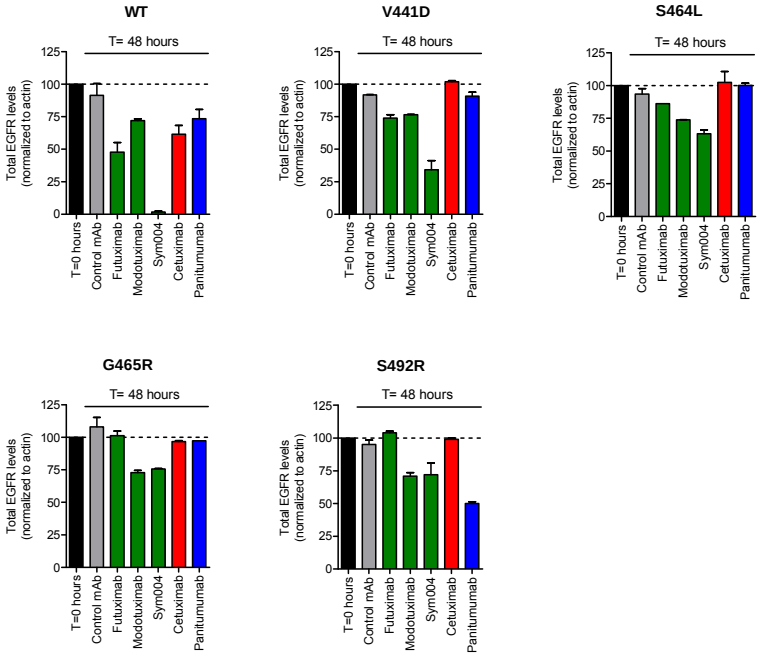
eFigure 7. Dose-response curves showing the effect of the indicated antibodies on cell viability in NIH- 3T3 cells stably overexpressing WT or mutant EGFR. Each data point represents the mean of three replicates ±SD.



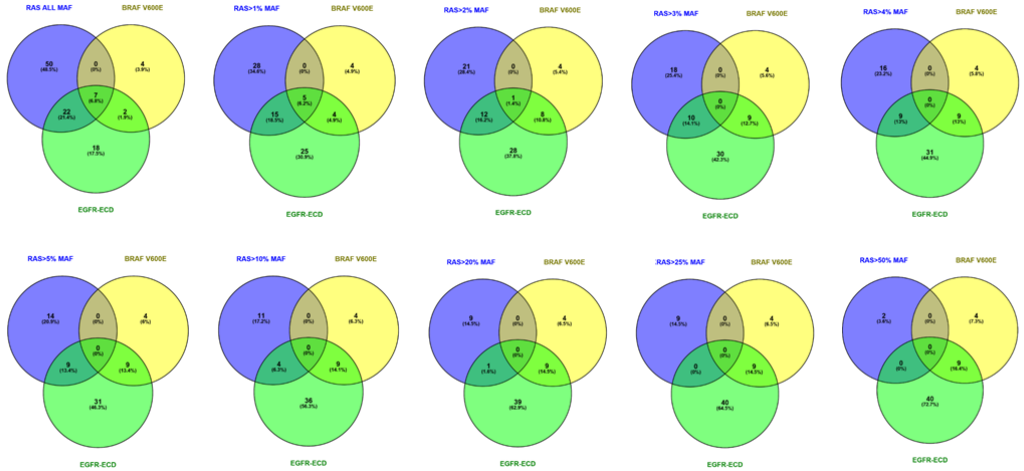
eFigure 8. Ability of Sym004 to block ligand induced phosphorylation of EGFR in NIH-3T3 cells transfected with either WT or mutant EGFR. Cells were cultured in the presence of the indicated drugs for 4 hours and stimulated with 1 nM EGF for 10 minutes. pEGFR (Tyr1068) levels were determined by Simple Western analysis. The pEGFR signal intensity was normalized to pan-actin (loading control) and is presented as a percentage of the signal in unstimulated control cells. Each bar represents the mean of three replicates. Error bars represent SD.



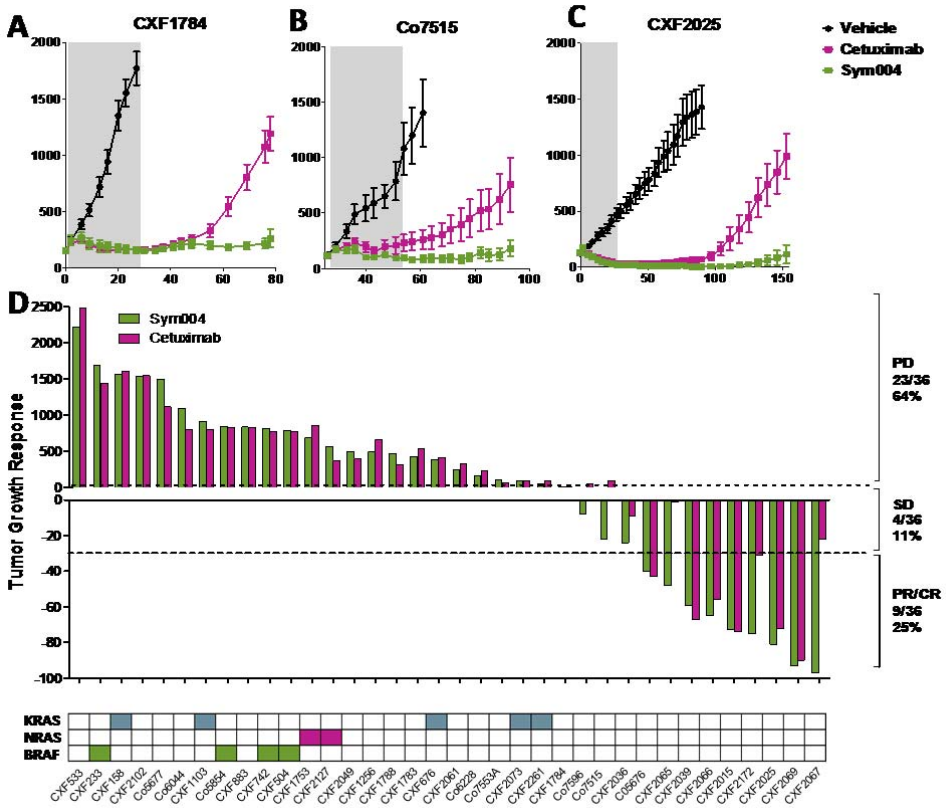
eFigure 9. Total EGFR levels after 48 hours of treatment with the indicated antibodies, as determined by Simple Western analysis. EGFR signal intensity was normalized to pan-actin (loading control) and is presented as a percentage of the signal in untreated control cells. Each bar represents the mean of three replicates. Error bars represent SD.



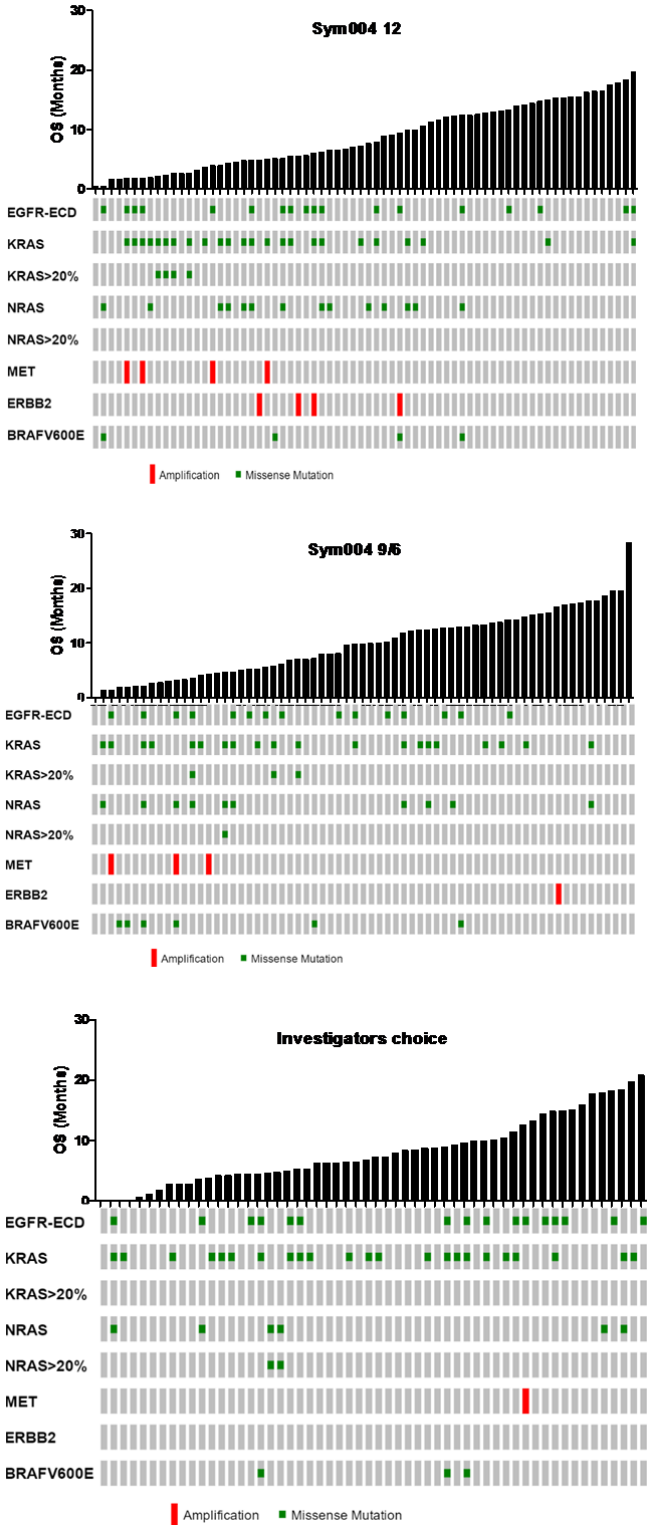
eFigure 10. Venn diagrams depicting the number (fraction of all profiled patients in parentheses) of patients harboring concurrent mutations in the EGFR ECD (G465E, G465R, S464L, S492R, V441D, and V441G) and KRAS/NRAS exons 2, 3, and 4 (RAS), as well as BRAF V600E, at various mutant allele frequencies (MAFs): RAS ALL MAF, RAS>1%MAF, RAS>2% MAF, RAS>3% MAF, RAS>4% MAF, RAS>5% MAF, RAS>10% MAF, RAS>20% MAF, RAS>25% MAF, and RAS>50% MAF.



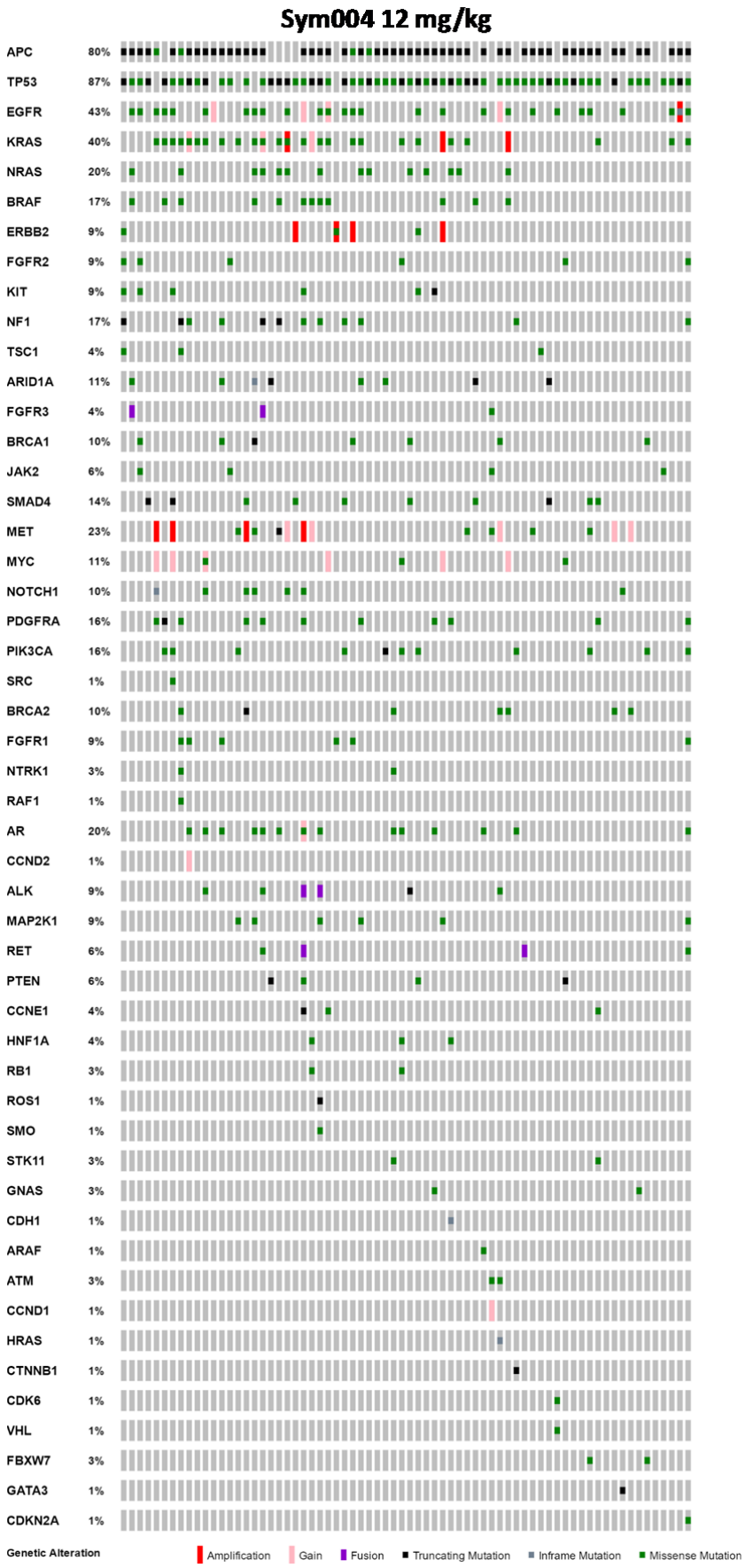
eFigure 11. (A), (B), and (C): Examples of tumor growth curves in PDX models. Animals were treated with vehicle (black), cetuximab (maroon), or Sym004 (green) (30 mg/kg i.p. twice weekly). The gray area marks the treatment period. (D) Waterfall plot showing tumor growth response at day 28, or the closest day to day 28, in 36 CRC PDX models treated with cetuximab (maroon) or Sym004 (green). PD: Progressive disease; SD: Stable disease; PR/CR: Partial response/complete response. (E) Mutations found in the PDX models: KRAS (green), NRAS (maroon), and BRAF (blue)

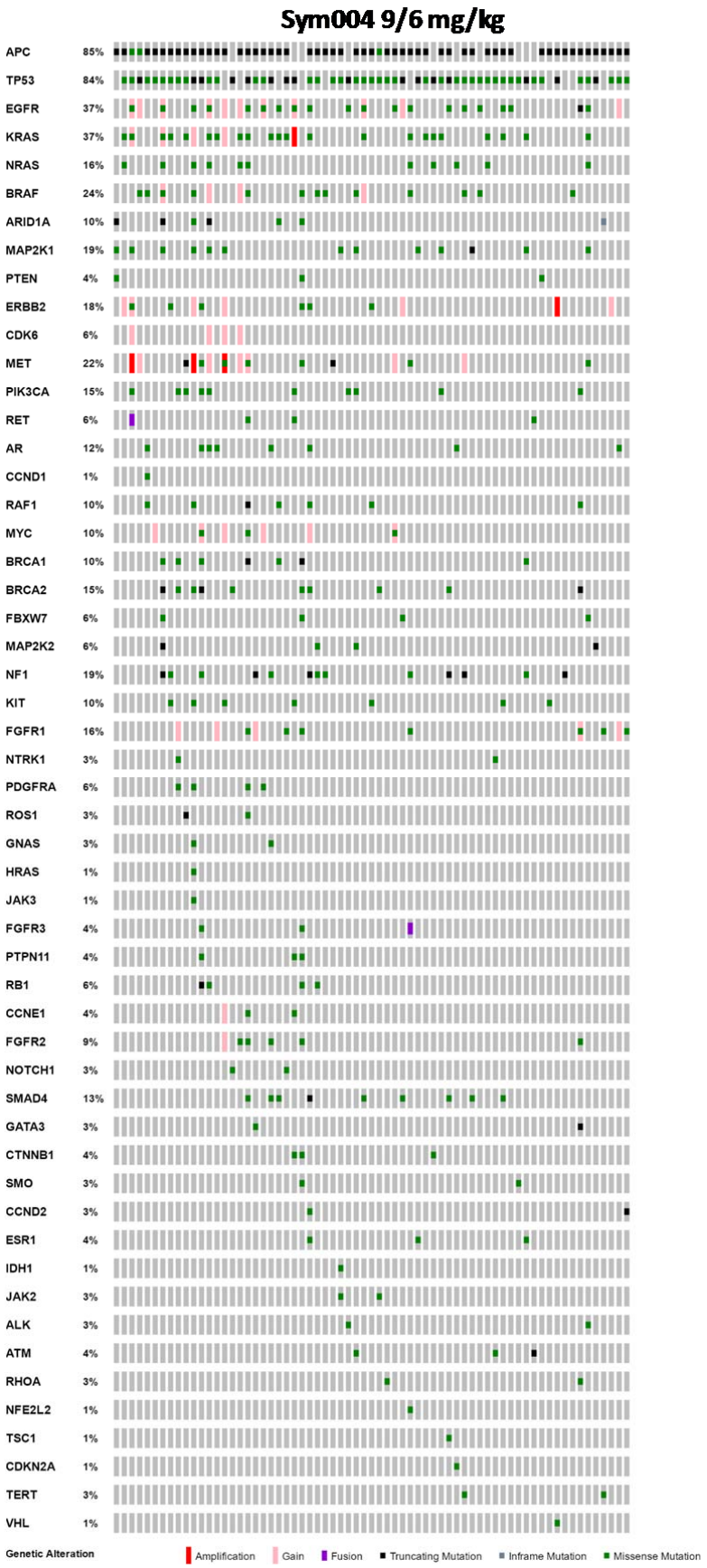


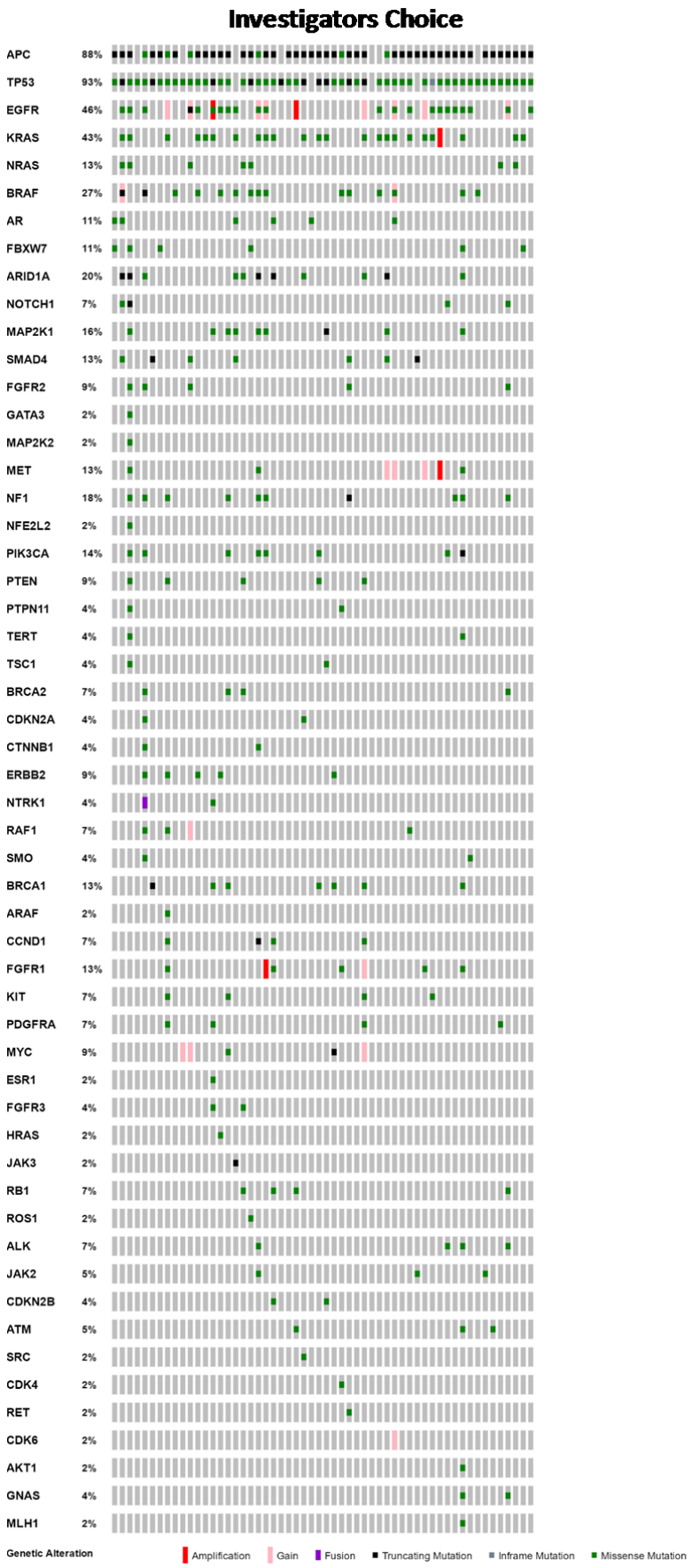
eFigure 12. Bar graphs depicting overall survival (OS) for each genetically profiled patient. Patients are grouped by treatment and sorted by increasing OS. The oncoprints denote patients with EGFR ECD mutations (G465R, G465E, S464L, S492R, V441D, and V441G), KRAS mutations in exon 2, 3, or 4 at all MAFs (KRAS) and at MAF>20% (KRAS MAF>20%), NRAS mutations in exon 2, 3, or 4 at all MAFs (NRAS) and at MAF>20% (NRAS MAF>20%), MET and ERBB2 gene amplifications (copy number >5), and BRAF V600E.



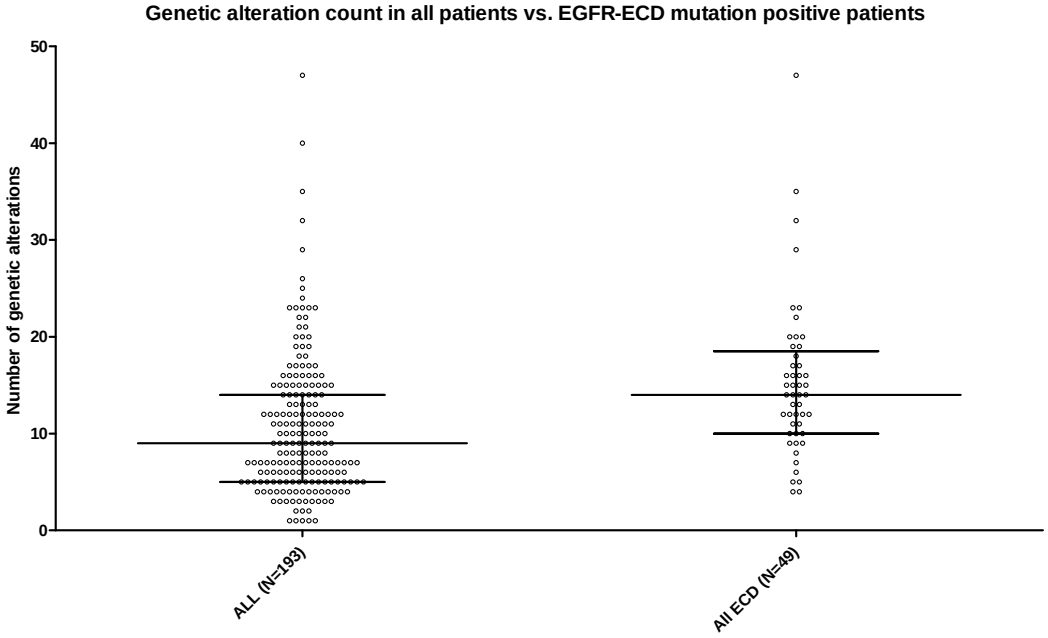
eFigure 13, 14 and 15. Oncoprints depicting the full ctDNA profiles of patients treated with Sym004 12 mg/kg (eFigure 13), Sym004 9/6 mg/kg (eFigure 14), or investigator's choice (eFigure 15). For all figures, the patients are sorted by overall survival, with poorest performing patients to the left. % denotes the fraction of patients in the treatment group with alterations in the specific gene. Amplifications are defined as more than five copies; gain is defined as copy number of more than 2.2 and less than five copies.







eFigure 16. Number of genetic alterations in all ctDNA profiled patients compared to patients harboring EGFR ECD mutations.



eTable 1. Response in ITT population (evaluable patients)

	Arm A Sym004 12 mg/kg (N=83)	Arm B Sym004 9/6 mg/kg (N=86)	Arm C Investigators Choice (N=85)
Best Overall Response – All Countries, n (%)			
CR	-	-	1 (1.4)
PR	11 (14.1)	8 (9.6)	1 (1.4)
SD	40 (51.3)	47 (56.6)	37 (52.9)
PD	27 (34.6)	28 (33.7)	31 (44.3)
Not evaluable	5	3	15
Disease Control Rate – All Countries, % (n/N evaluable)			
CR+PR+SD	65.4 (51/78)	66.2 (55/83)	55.7 (39/70)

eTable 2. Overall survival subsets analysis

ITT (N=254)	Sym004 12 mg/kg (N=83)	Sym004 9/6 mg/kg (N=86)	Investigator Choice (N=85)
mOS, months (95% CI)	7.9 (6.5, 9.9)	10.3 (9.0, 12.9)	9.6 (8.3, 12.2)
1-Year Survival Rate, %	37 (26, 47)	44 (33, 54)	40 (29, 51)
Hazard Ratio (95% CI)	1.31 (0.92, 1.87)	0.97 (0.68, 1.40)	
EU & US (N=224)	**Sym004 12 mg/kg (N=75)**	**Sym004 9/6 mg/kg (N=74)**	**Investigator Choice (N=75)**
mOS, months (95% CI)	7.7 (6.1, 11.3)	9.9 (8.0, 12.8)	8.5 (6.8, 10.2)
1-Year Survival Rate, %	38 (27, 49)	43 (31, 54)	34 (22, 45)
Hazard Ratio (95% CI)	1.09 (0.76, 1.58)	0.89 (0.61, 1.30)	
EU & US w. biomarker data (N=193)	**Sym004 12 mg/kg (N=70)**	**Sym004 9/6 mg/kg (N=67)**	**Investigator Choice (N=56)**
mOS, months (95% CI)	7.7 (5.5, 11.3)	9.9 (7.1, 12.9)	8.5 (6.4, 9.9)
1-Year Survival Rate, %	38 (26, 49)	44 (32, 56)	27 (16, 41)
Hazard Ratio (95% CI)	1.03 (0.69, 1.54)	0.79 (0.52, 1.20)	

eTable 3. Incidence of treatment emergent adverse effects (TEAE)

	Arm A Sym004 12 mg/kg (N_T[a] =83)	Arm B Sym004 9/6 mg/kg (N_T=84)	Arm C Investigators Choice (N_T=78)
Any TEAE	83 (100)	84 (100)	67 (85.9)
Any Related TEAE	81 (97.6)	80 (95.2)	46 (59.0)
Serious TEAE	27 (32.5)	23 (27.4)	12 (15.4)
Serious Related TEAE	9 (10.8)	6 (7.1)	2 (2.6)
TEAE leading to dose reduction	29 (34.9)	17 (20.2)	8 (10.3)
TEAE leading to study treatment discontinuation	12 (14.5)	5 (6.0)	6 (7.7)
Related TEAE leading to study treatment discontinuation	9 (10.8)	2 (2.4)	3 (3.8)
TEAE of Grade ≥3	67 (80.7)	53 (63.1)	25 (32.1)
Related TEAE of Grade ≥3	58 (69.9)	41 (48.8)	9 (11.5)
TEAE resulting in death	4 (4.8)	4 (4.8)	3 (3.8)
Related TEAE resulting in death	0	0	0
Dermatologic toxicity[b]	78 (94.0)	98 (92.9)	8 (10.3)
Dermatologic toxicity ≥3	45 (54.2)	31 (36.9)	1 (1.3)
Hypomagnesemia	57 (68.7)	47 (56.0)	6 (7.7)
Hypomagnesemia ≥3	27 (32.5)	14 (16.7)	0
Gastrointestinal disorders[c]	43 (51.8)	41 (48.8)	37 (47.4)
Gastrointestinal disorders ≥3	13 (15.7)	6 (7.1)	6 (7.7)
Infections and infestations	41 (49.4)	39 (46.4)	11 (14.1)
Infections and infestations ≥3	8 (9.6)	8 (9.5)	2 (2.6)
Infusion reaction	20 (24.1)	15 (17.9)	0
Hypokalemia	10 (12.0)	4 (4.8)	3 (3.8)

[a]Number of patients who received study treatment.[b]Dermatologic toxicity includes any AE terms described in the “Dermatologic Toxicity” sections in the 2015 package inserts for cetuximab, panitumumab, or necitumumab, as well as AE terms under infectious sequelae in the cetuximab package insert unless all AEs with a specific Preferred Term are unrelated. [c]Gastrointestinal disorders include all AEs under the MedDRA System Organ Class.

eTable 4. Baseline characteristics of DNmCRC and TNmCRC populations

DNmCRC[a]		Arm A Sym004 12 mg/kg (N=60)	Arm B Sym004 9/6 mg/kg (N=57)	Arm C Investigators Choice (N=51)
Age mean ± s.d.[b], years		63 ± 10.1	65 ± 10.7	62 ± 11.2
Sex, N (%)	Male	43 (69.4)	37 (64.9)	33 (64.7)
	Female	19 (30.6)	20 (35.1)	18 (35.3)
Race, N (%)	White	52 (83.9)	48 (84.2)	42 (82.4)
	Other or N/A	10 (16.1)	9 (15.8)	9 (17.6)
ECOG PS, N (%)	0	28 (45.2)	28 (49.1)	26 (51.0)
	1	34 (54.8)	28 (49.1)	25 (49.0)
	2	-	1 (1.8)[c]	-
Number of prior mCRC treatments[d], N (%)	2	14 (22.6)	6 (10.5)	10 (19.6)
	3	23 (37.1)	19 (33.3)	18 (35.3)
	≥ 4	25 (40.3)	32 (56.1)	23 (45.1)
Prior anti-EGFR mAb therapies, N (%)	Cetuximab only	42 (67.7)	33 (57.9)	28 (54.9)
	Cetuximab & Panitumumab	9 (14.5)	10 (17.5)	10 (19.6)
	Panitumumab only	11 (17.7)	14 (24.6)	13 (25.5)
Time since last anti-EGFR mAb therapy, days mean ± s.d.		81 ± 49.7	81 ± 52.8	69 ± 44.2
TNmCRC[e]		**Sym004 12 mg/kg (N=47)**	**Sym004 9/6 mg/kg (N=46)**	**Investigators Choice (N=38)**
Age mean ± s.d., years		63 ± 10.0	65 ± 9.3	63 ± 12.0
Sex, N (%)	Male	35 (74.5)	29 (63.0)	21 (55.3)
	Female	12 (25.5)	17 (37.0)	17 (44.7)
Race, N (%)	White	40 (85.1)	40 (87.0)	32 (84.2)
	Other or N/A	7 (14.9)	6 (13.0)	6 (15.8)
ECOG PS, N (%)	0	27 (57.4)	24 (52.2)	17 (44.7)
	1	20 (42.6)	21 (45.7)	21 (55.3)
	2	-	1 (2.2)[c]	-
Number of prior mCRC treatments[d], N (%)	2	10 (21.3)	5 (10.9)	8 (21.1)
	3	20 (42.6)	17 (37.0)	14 (36.8)
	≥ 4	17 (36.2)	24 (52.2)	16 (42.1)
Prior anti-EGFR mAb therapies, N (%)	Cetuximab only	33 (70.2)	29 (63.0)	24 (63.2)
	Cetuximab & Panitumumab	7 (14.9)	6 (13.0)	6 (15.8)
	Panitumumab only	7 (14.9)	11 (23.9)	8 (21.1)
Time since last anti-EGFR mAb therapy, days mean ± s.d.		78 ± 49.3	79 ± 52.2	65 ± 40.5

EFFICACY DATA, Overall Survival, Months	**Sym004 12 mg/kg**	**Sym004 9/6 mg/kg**	**Investigators Choice**
Population per Protocol with DNmCRC, N=170 (88%)	8.9 (6.2, 12.4) N=62	11.9 (9.7, 13.8) N=57	8.4 (6.4, 10.0) N=51
Population per Protocol with TNmCRC, N=131 (68%)	10.6 (6.8, 13.1) N=47	12.8 (9.7, 14.7) N=46	7.3 (6.3, 8.8) N=38

[a] DNmCRC: Double Negative metastatic Colorectal Cancer. [b] Standard deviation. [c] Patient had an ECOG performance status of 1 at screening and therefore met eligibility criteria. [d] All patients received at least one anti-EGFR antibody containing prior cancer therapy. [e] TNmCRC: Triple Negative metastatic Colorectal Cancer.