

Mutation status of KRAS, BRAF and PIK3CA combined with high expression of AREG and EREG predict response to cetuximab in a large panel of patient-derived colon carcinoma xenografts of all four UICC stages

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Introduction and Aim

Despite the large number of novel targeted cancer drugs that entered preclinical and clinical development, only a few of them turned out to be efficacious and were approved. A major reason for failure is the discrepancy between current preclinical testing modalities and the heterogeneity and diversity of human cancers. Limited opportunities in clinical biomarker development also contribute to the high attrition rate. To close this gap in colorectal cancer (CRC) we developed a large panel of 133 xenograft models derived from fresh tumor specimens of CRC patients across all four stages. This collection allows to mimic randomized phase II trials and to test effectively novel drugs as single agents or in combinations. It also enables the development of highly accurate companion diagnostics as demonstrated by us for cetuximab.

Patients

All fresh human tumor tissue samples originated from a multicenter prospective study "Molecular Signatures in Colorectal Cancer" (MSKK). 239 tumor tissue samples from patients of all four UICC stages were collected during two years by a collaborating network of four clinics, using a standardized procedure. In parallel, comprehensive clinical data were collected for each patient and monitored. All patients gave written informed consent prior to surgery. Clinical characteristics of 67 chemo-naïve patients with primary CRC are listed in Tab. 2.

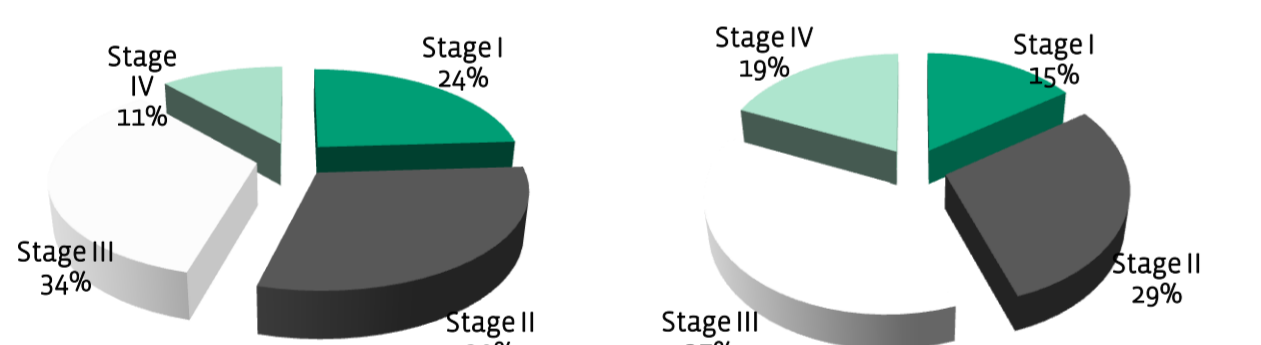


Fig. 1. Stage distribution in A) 4468 patients of the MSKK study; B) set of the 133 patient-derived xenograft models

Xenografts Establishment

Shortly after surgery, original tumor pieces were transplanted onto immunodeficient mice** and were further passaged until a stable growing tumor xenograft developed. In this way a panel of 149 passagable colon cancer xenografts was established (engraftment rate of 62%). After pathological evaluation 133 xenograft models remained (see Fig. 2).

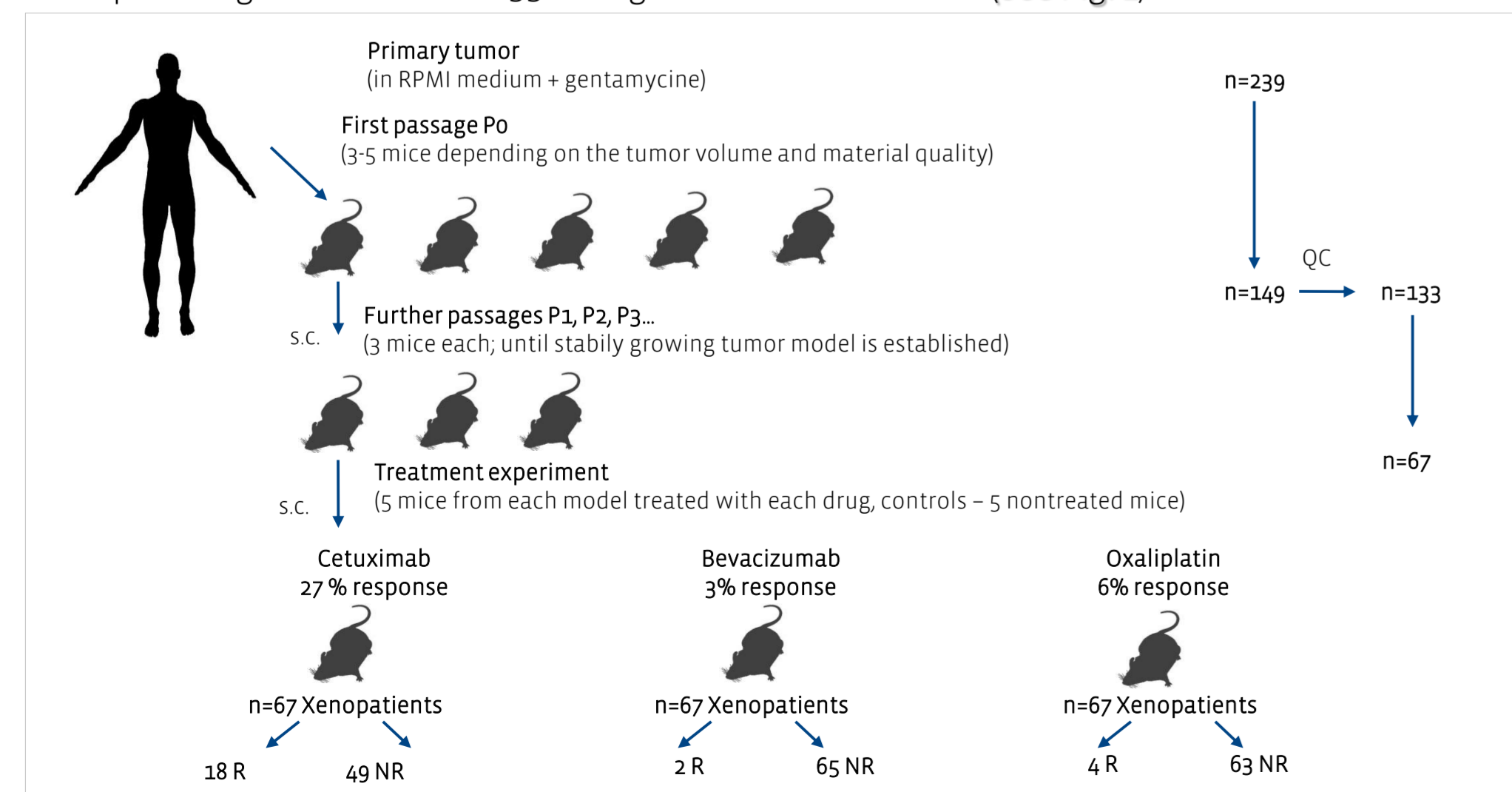


Fig. 2. Workflow scheme of transplantation of the 239 CRC patients tumor tissues, engraftment of 149 and treatment of 75 of the engrafted models with cetuximab, bevacizumab and oxaliplatin; s.c. - sterility check; QC - quality control

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Treatment Experiment

In three separate therapy experiments, 67 of the 133 available xenograft models were treated with cetuximab, bevacizumab and oxaliplatin as single-agents (Tab. 1). Each of the 67 tumors was transplanted onto 20 mice (5 controls and 5 for each drug). Responder models were defined with treated-to-control (T/C) ratios (volume of the treated tumor in relation to the non-treated control) of <20%. Nonresponder (resistant) models were defined with T/C ratios greater than 20%. Eighteen of 67 models showed a strong response to cetuximab monotherapy. In contrast, only 4 (6%) models responded to oxaliplatin and 2 models (3%) to bevacizumab (Fig. 1, Tab. 1).

Tab. 1. Treatment scheme and response rates of the 67 xenograft models. Treatment groups consist of 5 animals each. T/C values represent the treated-to-control ratios of relative median tumor volumes.

Drug	Treatment scheme	Responder T/C < 20% (> 80% tumour growth inhibition)	
		Number	%
Oxaliplatin (Eloxatin®, Sanofi-Aventis)	qd 1-5; 5 mg/kg/d, i.p.	4/67	6%
Cetuximab (Erbix®; Merck)	qd 7x2; 50 mg/kg/d, i.v.	18/67	27%
Bevacizumab (Avastin®; Genentech Inc.)	qd 4; 5 mg/kg/d, i.p.	2/67	3%

Mutation Analysis

Mutation analysis was performed by allele-specific RT-PCR (Custom TaqMan SNP Genotyping Assays, Applied Biosystems). TaqManMGB assays were designed to detect as follows: 8 substitutions in the KRAS gene (G12S, G12C, G12R, G12D, G12V, G12A, G13D, and A146T); the most frequent mutation in the BRAF gene (V600E); and 3 mutations in the PIK3CA gene (E542K, E545K, H1047R). As positive controls for the TaqManMGB assays we used 13 different cancer cell lines. Mutations in codon 61 of the KRAS gene were analyzed by direct sequencing in the samples that showed no mutation in codon 12, 13 and 146.

Altogether we observed at least one mutation in 41 of the 67 (61%) xenograft models (Fig. 3). There were 32 single mutations: 25 in KRAS, 6 in BRAF and 1 in PIK3CA. In 9 of the 67 (13%) CRC models we observed mutations in two genes, 8 in KRAS and PIK3CA and 1 in BRAF and PIK3CA. KRAS and BRAF mutations were mutually exclusive.

The corresponding primary patient tumors showed the same mutation profile.

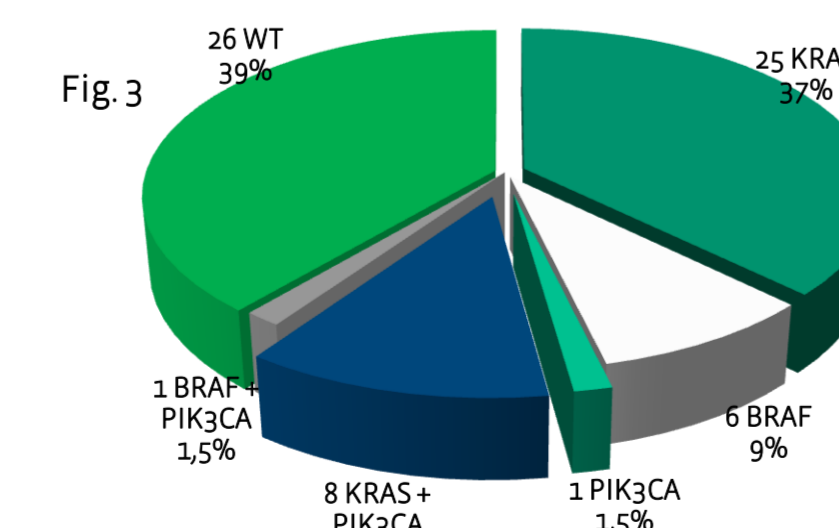


Fig. 3. Mutation distribution in 67 xenograft models (left)

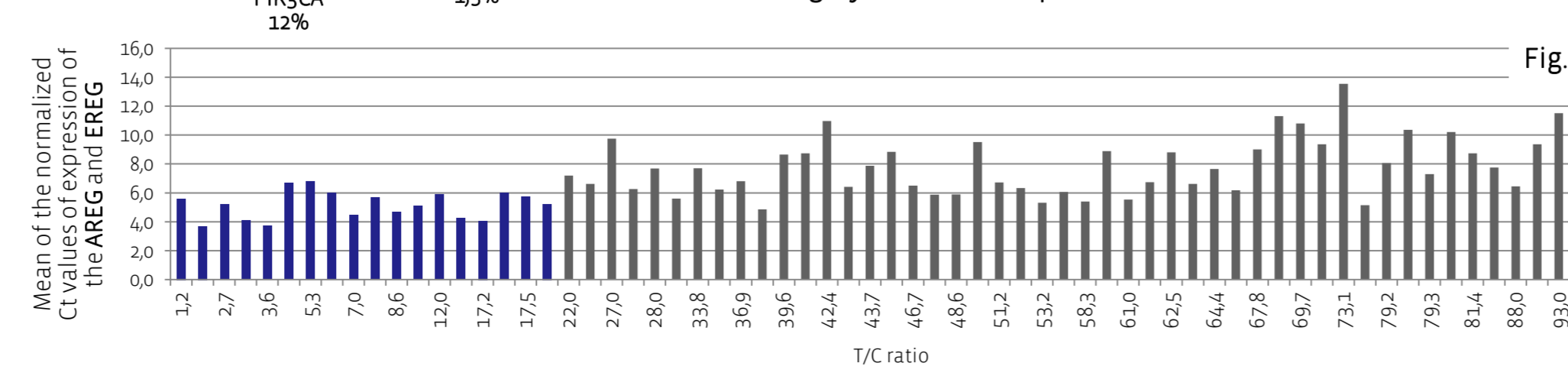
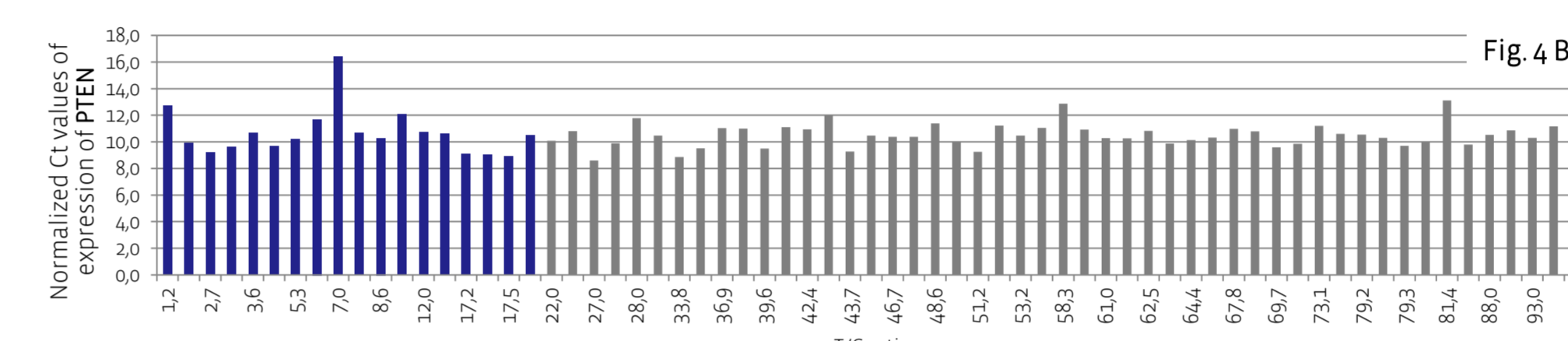


Fig. 4. Relation of the T/C ratio to the expression values shown as normalized Ct values (inverse relation to the gene expression) of selected genes; A) Mean of AREG and EREG and B) PTEN in a set of 67 treated xenograft models; blue bars - responders; grey bars - nonresponders

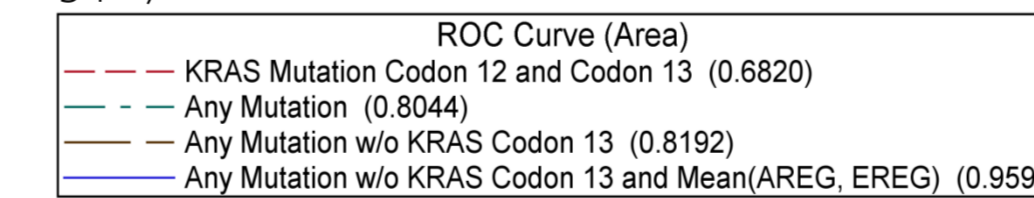


Tab. 2. Mutation distribution in a group of 67 xenograft models in respect to the patient characteristics

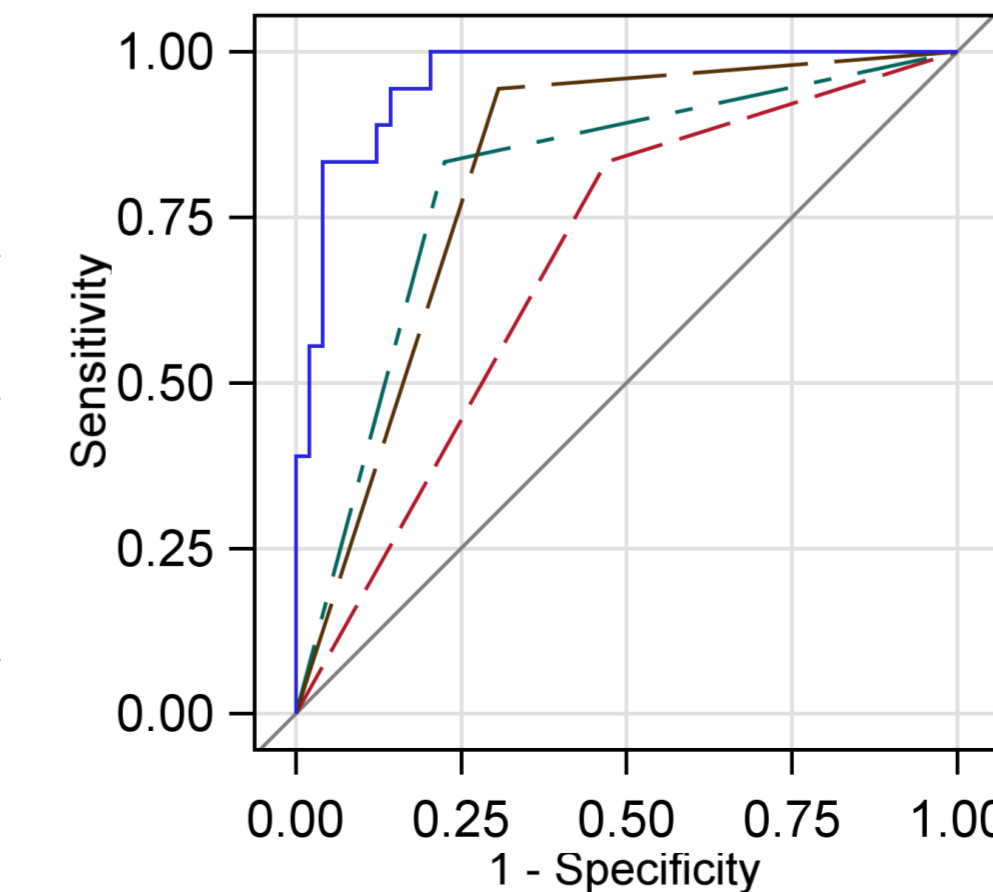
Characteristic	Total	BRAF Mut	%	KRAS Mut	%	PIK3CA Mut	%	Double Mut	%	Resp.	%
Total n pts	67	7	10%	33	49%	10	15%	9	13%	18	27%
Gender											
Male	31	2	6%	14	45%	4	13%	4	13%	9	29%
Female	36	5	14%	19	53%	6	17%	5	14%	9	25%
Age											
≤60 yrs old	15	0	-	7	47%	2	13%	2	13%	4	27%
>60 yrs old	52	7	13%	26	50%	8	15%	7	13%	14	27%
Location											
Colon	46	7	15%	20	43%	9	20%	8	17%	10	22%
Rectum	21	0	-	13	62%	1	5%	1	5%	8	38%
UICC Stage											
I	8	1	13%	5	63%	2	25%	2	25%	2	25%
II	22	2	9%	7	32%	1	5%	1	5%	8	36%
III	28	3	11%	18	64%	6	21%	5	18%	4	14%
IV	9	1	11%	3	33%	1	11%	1	11%	4	44%

Gene Expression Analysis

RNA expression of AREG, EREG, PTEN, DUSP6 and SLC26A3 was analyzed by TaqMan real-time PCR and normalized toward three housekeeping genes GAPDH, RPLPo and UBC. A significant correlation was found between high expression of AREG and EREG (low Ct values) and response to cetuximab (Fig. 4A). The Pearson Correlation Coefficient (PCC) of AREG and EREG was 58% and 52%, respectively. PTEN expression was not correlated with cetuximab response (PCC=6%, Fig. 4B), while DUSP6 and SLC26A3 showed a weak correlation (PCC=31% and 34%).



We constructed ROC curves for four different predictors that include: 1: KRAS codon 12 and 13 mutations (dashed red); 2: All KRAS, BRAF and PIK3CA mutations (dashed green); 3: KRAS, BRAF and PIK3CA excluding KRAS codon 13 mutations (dashed brown); 4: KRAS excluding codon 13, BRAF and PIK3CA mutations combined with AREG and EREG RNA expression (blue). The area under the curve (AUC) was: 0.68, 0.80, 0.82 and 0.96 for the four different predictors respectively (see Fig. 5, right).



Predicting Response to Cetuximab

Applying the standard approach of KRAS codon 12 and 13 testing in our set of 67 models, 15 of them would be correctly treated and 23 patients would be over-treated without benefit from the anti EGFR treatment. This is reflected by the positive predictive value (PPV) of only 15/38 = 0.39 and a negative predictive value (NPV) of 26/29 = 0.90 (Tab. 4, Predictor 1).

If testing involves also KRAS codon 61 and 146 and BRAF and PIK3CA mutations (Tab. 4, Predictor 2) 15 patients would be treated correctly, 11 of the 26 wildtype patients would be over-treated and would not benefit from the anti-EGFR treatment (PPV: 15/26 = 0.58, NPV: 38/41 = 0.93).

If the KRAS codon 13 mutations are counted as responders (remaining parameters -Predictor 2), the PPV of is only 17/32 = 0.53 while the NPV would increase to 34/35 = 0.97 (Tab. 4 Predictor 3).

The most powerful predictor combines KRAS, BRAF, and PIK3CA mutations and RNA expression of AREG and EREG. Among the 24 patients predicted to respond there are 17 responders and 7 nonresponder (PPV: 17/24 = 0.71). Of the 43 patients predicted to be resistant one patient would be a true responder (NPV: 42/43 = 0.98) (Tab. 4 Predictor 4).

Tab. 4. Cross tabulation table shows the relation between response towards cetuximab and: Predictor 1: Mutation status in codon 12 and 13 of the KRAS (standard mutation detection procedure); Predictor 2: All analyzed mutation in KRAS, BRAF and PIK3CA gene; Predictor 3: by mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA; Predictor 4: by mutations in KRAS (excluding codon 13 mutations), BRAF and PIK3CA, combined with RNA expression of AREG and EREG.

Predictor	Predicted response (wildtype)	Observed Response		Sums:
		Yes	No	
Predictor 1	Yes	15	23	38
	No	3	26	29
	Sums:	18	49	67
Sensitivity/Specificity:		83%(61%-94%)	53%(39%-66%)	
Predictor 2	Yes	15	11	26
	No	3	38	41
	Sums:	18	49	67
Sensitivity/Specificity:		83%(61%-94%)	78%(64%-87%)	
Predictor 3	Yes (+ codon 13)	17	15	32
	No	1	34	35
	Sums:	18	49	67
Sensitivity/Specificity:		94%(74%-99%)	69%(55%-80%)	
Predictor 4	Yes (+ codon 13)	17	7	24
	No	1	42	43
	Sums:	18	49	67
Sensitivity/Specificity:		94%(74%-99%)	86%(73%-93%)	

Conclusions

To advance preclinical testing of novel targeted drugs and predictive biomarkers a panel of 133 mouse xenograft models from 239 fresh primary tumor specimens of patients with CRC of all four UICC stages was established.

A set of 67 xenograft models was treated with cetuximab, bevacizumab and oxaliplatin as single agents. We observed response rates of 27% (18/67) for cetuximab, 3% (2/67) for bevacizumab, and 6% (4/67) for oxaliplatin.

A highly accurate companion diagnostics that combined KRAS, BRAF, and PIK3CA mutations status with amphiregulin and epiregulin RNA expression predicts response to anti-EGFR treatment with a sensitivity of 94%, a specificity of 86% and an AUC of 0.96.

CDx could be valuable for stratification of patients with mCRC but also for patients with stage II and III disease in the adjuvant setting after validation.

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