Circulating tumour DNA sequencing identifies a genetic resistance-gap in colorectal cancers with acquired resistance to EGFR-antibodies and chemotherapy

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Abstract: Epidermal growth factor receptor antibodies (EGFR-Abs) confer survival benefit in patients with *RAS* wild-type metastatic colorectal cancer (mCRC) but resistance invariably occurs. Previous data showed that only a minority of cancer cells harboured known genetic resistance drivers at the time clinical resistance to single-agent EGFR-Abs had evolved, supporting the activity of non-genetic resistance mechanisms. Here, we used error-corrected ctDNA-sequencing (ctDNA-Seq) of 40 cancer genes to identify drivers of resistance and whether a genetic resistance-gap (a lack of detectable genetic resistance mechanisms in a large fraction of the cancer cell population) also occurs in *RAS* wild-type mCRCs treated with a combination of EGFR-Abs and chemotherapy. We detected one *MAP2K1/MEK1* mutation and one *ERBB2* amplification in 2/3 patients with primary resistance and *KRAS, NRAS, MAP2K1/MEK1* mutations and *ERBB2* aberrations in 6/7 patients with acquired resistance. *In vitro* testing identified MAP2K1/MEK1 P124S as a novel driver of EGFR-Ab resistance. Mutation subclonality analyses confirmed a genetic resistance-gap in mCRCs treated with EGFR-Abs and chemotherapy, with only 13.42% of cancer cells harboring identifiable resistance drivers. Our results support the utility of ctDNA-Seq to guide treatment allocation for patients with resistance and the importance to further investigate non-canonical EGFR-Ab resistance mechanisms, such as microenvironmentally-mediated resistance. The detection of *MAP2K1* mutations could inform trials of MEK-inhibitors in these tumours.

Keywords: colorectal cancer, ctDNA-Sequencing, ctDNA-ddPCR, acquired resistance, genetic resistance-gap, EGFR-antibodies

1. Introduction

KRAS and *NRAS* mutations are predictors of primary resistance to the EGFR antibodies (EGFR-Abs) cetuximab and panitumumab in metastatic colorectal cancer (mCRC) [1-5]. Furthermore, *RAS* mutations evolve in most mCRCs at the time they acquire resistance to EGFR-Abs [6-8]. Other genetic aberrations that re-activate the RAS/RAF pathway such as *EGFR* and *BRAF* mutations or *ERBB2* amplifications also confer primary and acquired resistance but are less common [9,10]. Analysing the mutation status of these driver genes in the circulating tumour DNA (ctDNA) through so-called 'liquid biopsies' can avoid the need for tumour re-biopsies, which are associated with discomfort, a risk of complications and high costs. Furthermore, early detection of evolving resistance drivers may help to monitor patients and to guide personalized treatment switching to alternative therapies.

Application of liquid biopsies in mCRC patient's management is becoming increasingly feasible through the development of ctDNA-sequencing (ctDNA-Seq) technologies incorporating error correction [11,12], which enable mutation detection in entire gene panels with high sensitivity and low false positive rates. We developed a ctDNA-Seq assay for CRC patients that applies molecular barcodes (MBC) and duplex DNA identification for error correction and can be performed from 25ng of ctDNA. We showed that this could call mutations with variant allele frequencies (VAFs) of 0.15% in ctDNA [12].

Application of ctDNA-Seq to *RAS* wt mCRC patients who acquired resistance to single-agent cetuximab in the third line setting showed the ability to identify mutations and DNA amplifications that drive resistance [9]. Leveraging the ability of this ctDNA-Seq technique to reconstruct genome wide copy number profiles [12], we assessed the clonality of resistance driver mutations by first correcting VAFs for the influence of copy-number states and by subsequently calculating the proportion of cancer cells that harbored resistance driver mutations by comparing against *TP53* or *APC* mutations, which are likely clonal. This subclonality analysis revealed that only a minority (36%) of cancer cells represented in the ctDNA did harbour resistance driver mutations despite radiological progression. This defined a previously undiscovered genetic resistance-gap at the time of acquired cetuximab resistance and led to the

discovered of a novel non-genetic mechanism of single-agent cetuximab resistance, driven by an increase in tumour associated fibroblasts [9].

In this study, we first aimed to validate the ability of this ctDNA-Seq technology using a targeted 40 gene panel to identify mutations in 10 patients who initially showed *RAS* wild-type status in tumour tissue and either showed primary or acquired resistance when treated with EGFR-Ab therapy predominantly in combination with chemotherapy. Moreover, as most patients in this study received a combination of EGFR-Abs and chemotherapy, we investigated what proportion of the cancer cells harboured these drivers to assess if a genetic resistance-gap also occurs in mCRCs that acquired resistance to chemotherapy and EGFR-Ab or if this only arises with single-agent cetuximab.

2. Results

Plasma samples were collected after radiologically confirmed progression from ten patients with mCRCs that were *RAS* wild-type based on clinical testing (Table S1). Nine of them had received an EGFR-Ab (cetuximab or panitumumab) in combination with chemotherapy and one single-agent EGFR-Ab (panitumumab) (Table 1). Two (patients 3 and 4) were analyzed at the time they were re-challenged with EGFR-Ab therapy. Analogous to previous work [9], we classified patients with progressive disease (PD) within 12 weeks of EGFR-Ab initiation (n=3, median time to progression: 9 weeks) as cases with primary resistance. Those that obtained benefit for at least 12 weeks (n=7, median time to progression: 26 weeks) before they progressed were considered as cases with acquired resistance.

Table 1. Clinical characterists of metastatic colorectal cancer patients.

Patient ID	Age (years)	Gender	Histology	Primary location	Differentiation grade	EGFR-Ab therapy	Line of therapy for metastatic disease	Time on EGFR-Ab therapy	Resistance
1	80	Male	Adenocarcinoma	Right-colon	Moderate	Panitumumab + FOLFOX	2nd	2 weeks	Primary
2	79	Male	Adenocarcinoma	Rectum	Moderate	Panitumumab	3rd	9 weeks	Primary
3	57	Male	Adenocarcinoma	Sigmoid	Well	Cetuximab + Irinotecan (rechallenge with EGFR-Ab)	3rd	10 weeks	Primary
4	58	Female	Adenocarcinoma	Rectum	Well	Cetuximab + Irinotecan (rechallenge with EGFR-Ab)	3rd	16 weeks	Acquired
5	52	Male	Adenocarcinoma	Sigmoid	Moderate	Cetuximab + Irinotecan	2nd	20 weeks	Acquired
6	64	Male	Adenocarcinoma	Rectum	Moderate	Panitumumab + FOLFOX	1st	12 weeks	Acquired
7	41	Female	Adenocarcinoma	Sigmoid	Poor	Cetuximab + FOLFIRI	1st	27 weeks	Acquired
8	53	Female	Adenocarcinoma	Right-colon	Poor	Cetuximab + FOLFIRI	2nd	27 weeks	Acquired
9	46	Female	Adenocarcinoma	Sigmoid	Moderate	Panitumumab + FOLFOX	2nd	29 weeks	Acquired
10	30	Male	Adenocarcinoma	Rectum	Moderate	Panitumumab + FOLFIRI	5th	26 weeks	Acquired

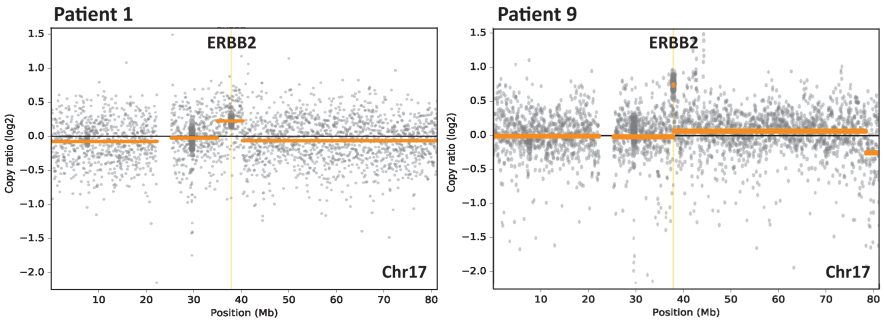
2.1 ctDNA sequencing results

Up to 25ng of the ctDNA were sequenced with our error-corrected ctDNA-Seq panel (40 cancer genes, 221kb target region), which includes commonly mutated CRC driver genes (*APC, TP53, FBXW7, PIK3CA and SMAD2/4)* and known EGFR-Ab resistance driver genes (*KRAS*, *NRAS*, *EGFR*, *BRAF*, *MAP2K1*, *MET, NF1, FGFR2* and *ERBB2*) [1-10]. The average read depth in the analyzable target region after MBC deduplication was 1388x (Figure 1A). Mutations in the CRC driver genes *TP53* or *APC* were identified in the ctDNA of 9 out of 10 patients (Figure 1A). Genome-wide DNA copy number profiles were reconstructed for all cases to identify gene amplifications (Figure S1).

A

Patient ID	Average Depth - Seq	*TP53*	AA change	*APC*	AA change
1	1756	3.23%	R248Q	1.0%; 2.67%	M891Kfs*27; I1418Yfs*5
2	758	-	-	0.67%	S1344*
3	821	-	-	0.53%	R1450*
4	1479	42.86%	Y163Sfs*8	26.92%; 41.60%	S1398Efs*11; p.R858*
5	2429	1.17%	A129Cfs*20	1.32%	R805*
6	1549	28.89%	P278A	17.31%	E1309Dfs*4
7	2260	2.49%	P191del	-	-
8	950	6.28%; 6.56%	R280G; R273H	-	-
9	1348	1.01%	Q192*	1.33%	Q1367*
10	525	-	-	-	-

B **C**



D

	Patient ID	Therapy	KRAS G12D	KRAS G13F	KRAS Q61H	NRAS G12S	NRAS G13D	EGFR K467E	MAP2K1 K57N	MAP2K1 K57T	MAP2K1 P124S	ERBB2 R143Q	ERBB2
Primary resistance	1	Panitumuab + FOLFOX											amp
	2	Panitumumab							0.47%				
	3	Cetuximab + Irinotecan											
Acquired resistance	4	Cetuximab + Irinotecan					0.49%	1.11%					
	5	Cetuximab + Irinotecan									0.10%		
	6	Panitumuab + FOLFOX			0.11%	0.68%				0.25%			
	7	Cetuximab + FOLFIRI										0.19%	
	8	Cetuximab + FOLFIRI		0.28%									
	9	Panitumumab + FOLFOX											amp
	10	Panitumumab + FOLFIRI											

Figure 1. Resistance drivers identified by ctDNA-Seq in mCRC patients at PD to anti-EGFR-Abs. **(A)** Non-silent mutations in the CRC driver genes *TP53* or *APC* identified in the ctDNA and average read depth in ctDNA-Seq. The Variant Allele Frequencies for each mutation are shown. **(B)** Chromosome 17 copy number profile for patient 1 **(C)** and for patient 9. **(D)** Drivers mutations/amplifications identified by ctDNA-Seq. Numbers represent the Variant Allele Frequencies of detected mutations.

2.1.1 Identification of drivers of primary resistance by ctDNA sequencing

We next identified likely drivers of resistance to EGFR-Ab in the three patients with primary resistant mCRCs. An *ERBB2* amplification was detected in the copy number profile of patient 1 (Figure 1B). *ERBB2* amplifications have previously been shown to confer primary EGFR-Ab resistance [13]. Furthermore, a mutation in the tumour-suppressor gene *NF1* (F1247L) was called in this sample but this has not been seen in the Cosmic cancer mutation database and it was not an inactivating mutation (Table S2). No further resistance driver mutations were detected in this patient. In addition,

we identified a *MAP2K1/MEK1* K57N mutation in patient 2 (Figure 1D). K57N is known to constitutively activate MEK1 in colorectal cancer cell lines [14] and we and others previously showed a role in EGFR-Ab resistance [9,13]. No resistance mechanism was identified in patient 3. Thus, ctDNA-Seq identified an explanation for primary resistance in 2/3 cases (67%).

2.1.2 Identification of drivers of acquired resistance by ctDNA sequencing

We then analysed ctDNA-Seq results from the 7 patients with acquired EGFR-Ab resistance. Genetic aberrations that were likely responsible for acquired resistance were detected in 6/7 patients (Figure 1D). Two patients harboured more than one aberration. *NRAS* G13D and *EGFR* K467E mutations were found in patient 4, in addition to a *NF1* A2511V mutation reported in ClinVar as likely benign [15] (Table S2). *NRAS* G12S, *KRAS* Q61H and *MAP2K1/MEK1* K57T mutations were detected in patient 6. A *KRAS* G13F mutation was identified in patient 8 and a *MAP2K1/MEK1* P124S mutation in patient 5 (Figure 1D). P124S is located in the *MEK1* protein kinase domain and has previously been showed to confer resistance to BRAF- and MEK-inhibitor therapy in melanoma [16] but its role in EGFR-Ab resistance in CRC was unknown. Expression of *MAP2K1/MEK1* P124S and wild-type *MAP2K1/MEK1* in the cetuximab sensitive CRC cell line DiFi showed that the mutation rescued ERK phosphorylation and confirmed it as a new driver of acquired cetuximab resistance (Figure 2).

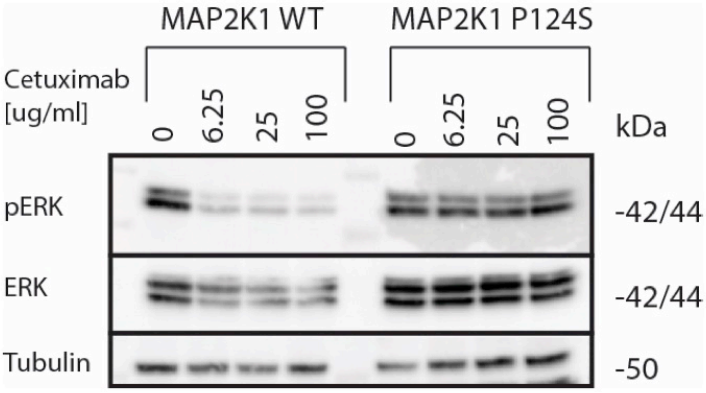


Figure 2. Western blot analysis of parental, *MAP2K1*/*MEK1* wild-type transduced and *MAP2K1*/*MEK1* P124S transduced DiFi cell line treated with cetuximab for 2 hours.

An amplification of *ERBB2* was identified in patient 9 (Figure 1C) and an *ERBB2* R143Q mutation (0.19%) in patient 7 (Figure 1D). The latter has previously been described in bladder cancer cell lines as a potential activating mutation, which sensitizes to the pan-EGFR inhibitor lapatinib *in-vitro* [17]. No driver of acquired resistance was identified by ctDNA-Seq in patient 10 (Figure 1D). This is likely due to low tumour content in the ctDNA as indicated by the absence of clear DNA copy number aberrations in this sample (Figure S1) and of *APC* or *TP53* mutations, which had been detected in all other samples.

Together, likely drivers of acquired EGFR-Ab resistance were detected in 86% (6/7) of patients using ctDNA-Seq (Figure 1D). Consistent with prior studies that showed that acquired resistance is often polyclonal [8,9], more than one resistance driver was detected in 2/7 (29%) patients.

2.1.3 Clonality of drivers of primary and acquired resistance

We recently showed that the majority of cancer cells did not harbor any resistance mutations at the time CRCs acquired resistance and progressed on single-agent cetuximab [9]. Whether a similar genetic resistance-gap occurs at acquired resistance in mCRCs treated with a combination of chemotherapy and EGFR-Ab is unknown. Using our established method [9], we assessed the clonality of resistance driver mutations by first correcting VAFs for the influence copy-number states and by subsequently calculating the proportion of cancer cells that harbored resistance driver mutations by comparing against *TP53* or *APC* mutations, which are likely clonal (Table S3). This also corrects for variable tumour contents in different ctDNA samples. Clonality assessment was not possible for patient 9 where an amplification had been detected as the absolute number of amplified DNA copies in such subclones cannot be assessed, and for patient 10 where no resistance drivers were identified.

The 8 driver mutations found in the remaining five tumours with acquired resistance were only present in a median of 7.65% (range 1.14%-17.24%) of the cancer cells sampled by ctDNA-Seq, and were therefore subclonal (Table S3). When all the mutations in each individual patient were added together, still only a median of 13.42% (range 8.91%-17.24%) of all cancer cells represented in the ctDNA were

mutated (Figure 3A). In comparison, when we applied the same analysis to patient 2, which showed primary resistance and a *MAP2K1/MEK1* mutation, this was estimated to be present in 100% of the ctDNA and hence clonal (Figure 3A).

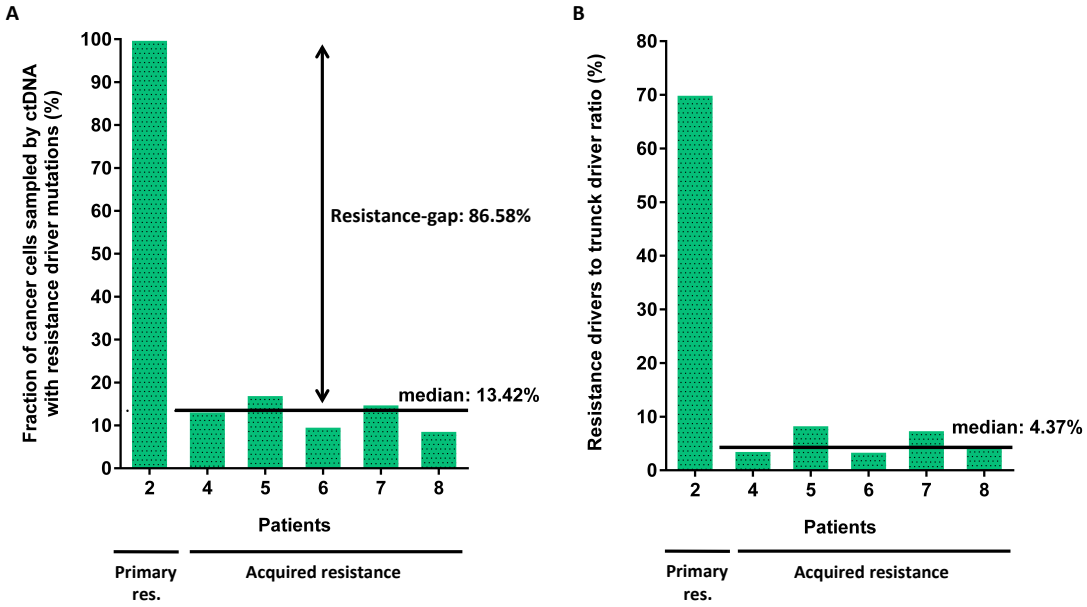


Figure 3. Clonality analysis of EGFR-Ab resistance driver mutations in ctDNA by comparison to truncal CRC driver mutations in *TP53* or *APC*. **(A)** Fraction of cancer cells sampled by ctDNA that harbored EGFR-Ab resistance driver mutations when VAFs are corrected for the influence of copy number aberrations. **(B)** Ratio of the VAF of all resistance drivers combined to the VAF of truncal mutations.

We used a conservative estimate to define the highest likely cancer cell fraction (see methods) but a potential limitation of this analysis is that the copy number states are estimates as allele specific copy number data cannot be generated from off-target reads, and this can lead to inaccuracies. We therefore also applied a published approach to estimate clonality, which uses the ratio of resistance mutation VAFs to the highest VAF of likely truncal drivers without any correction for copy number status [18]. All drivers of acquired resistance combined per case had a median ratio of 4.37% (range 3.60%-8.55%) compared to truncal mutations in either *TP53* or *APC* (Figure 3B). Thus, both approaches support the presence of a considerable genetic resistance-gap at acquired resistance to combination EGFR-Ab and chemotherapy.

3. Discussion

We identified *MAP2K1/MEK1* mutations in 3 and *RAS* mutations in 4 of 10 patients. *MAP2K1/MEK1* mutations were hence the second most common driver of resistance in this small series that was predominated by tumours with acquired resistance. While *MAP2K1/MEK1* codon K57 mutations have previously been associated with EGFR-Ab resistance [9,13,14] we provide the first evidence that P124S mutations contribute to resistance to EGFR-Ab therapy in mCRC. Together, our results highlight the importance to use ctDNA analysis panels that include a broad range of resistance driver genes beyond *RAS* and *BRAF* such as *MAP2K1* and *ERBB2* [12,19] to optimally stratify patients to EGFR-Abs. The detection of *ERBB2* amplifications and activating *MAPK2K1* through ctDNA-Seq could furthermore stratify these patients for treatment with trastuzumab or treatment with MEK-inhibitors in clinical trials [20]. The ability of ctDNA-Seq to assess mutation clonality may help to select tumours with clonal drivers to avoid targeting subclonal drivers which will likely be futile [21].

Importantly, subclonality analyses demonstrated that mutations driving acquired resistance to EGFR-Ab in combination with chemotherapy were confined to small subclones. No genetic resistance drivers were detected in a median of 86.58% of the cancer-derived ctDNA. This defines a genetic resistance-gap in patients with acquired resistance to chemotherapy and EGFR-Abs which is similar to the 64% of the cancer cells sampled by ctDNA had no detectable genetic resistance drivers observed in patients treated with single-agent cetuximab [9]. The clonality estimates are based on published approaches [9,18] but some inaccuracies are possible as these technologies are relatively novel and not all sources of bias may have been identified. Importantly, the average sequencing depth of our assay is similar to other current ctDNA sequencing technologies [22,23,24] and we have previously shown that the sensitivity of this assay is comparable to other technologies with error correction [12]. Thus, it is unlikely that poor assay sensitivity explains these results.

Moreover, we previously showed that tumours with a cetuximab-sensitive transcriptomic subtype before single-agent EGFR-Ab treatment changed to a fibroblast- and growth factor-rich subtype at progression and that this stromal remodeling enables non-genetic cetuximab resistance, likely explaining the genetic

resistance-gap [9]. Confirming a similar resistance-gap in mCRCs treated with EGFR-Ab and chemotherapy now suggests that non-genetic resistance mechanisms may also be relevant when combination therapy is used. This will require confirmation through studies of tumour biopsies in the future, particularly as several other candidate mechanisms for non-genetic resistance have been described, including myeloid derived suppressor cells infiltrates [25] or paracrine growth factor secretion by cancer cells [26]. Several of the non-genetic resistance mechanisms that were identified depend on secreted growth factors and may be clinically targetable through blocking agents. Dissection these mechanisms may therefore inform rational combination treatments with EGFR-Abs and chemotherapy. Minimally invasive technologies to assess the cancer microenvironment compositions or growth factor secretion in the microenvironment are an unmet need. Developing these could accelerate the interrogation of such understudied resistance mechanisms.

An alternative explanation for this resistance-gap could be that EGFR-Ab resistance is the consequence of genetic drivers scattered across a large number of genes that are rarely mutated individually and therefore remained unidentified to date. However, our previous finding that cancer associated fibroblasts increased in PD biopsies without detectable genetic resistance drivers, and that these can mechanistically rescue cancer cell growth, supported the non-genetic resistance model [9].

4. Materials and Methods

4.1 Patients

Ten patients with *RAS* wild-type status mCRCs who received treatment with EGFR-Ab (cetuximab/panitumumab) containing therapy were included in this study. The study has been approved by Hospital Sírio Libanês Ethics Committee (Study # HSL 2015-22) and all patients provided written informed consent before study inclusion. Information from clinical *RAS* mutation tests of tumour tissue form each patient was available for this study.

4.2 Plasma samples

Blood samples (15ml) were collected in EDTA-tubes at the time of clinical progression to EGFR-Ab. Plasma was separated by centrifugation at 800g for 10min at 4°C within 2h after collection. Plasma was spun again at 11,000g for 10min at 4°C and stored at −80°C. ctDNA was isolated using the QIAamp MinElute Virus Vacuum Kit (Qiagen).

4.3 ctDNA-Sequencing

Between 17.6ng and 25ng of ctDNA were sequenced per sample using Agilent SureSelect$^{XT\text{-}HS}$ library preparation and target enrichment of 40 genes as described [9,12]. Sequencing library pools were clustered using an Illumina cBot and sequenced with 75pb paired-end reads on an Illumina HiSeq2500 in rapid-output mode.

4.4 Variant calling

SureCall (version 4.0.1.46, Agilent) was used to trim and align fastq reads to the hg19 reference genome with default parameters and for Molecular Barcode error Correction (MBC) deduplication, permitting one base mismatch within each MBC. Consensus families comprising single reads were removed. The SureCall software (Agilent) was used to determine the average on-target read depth (the average number of reads at each position of the analyzable target regions) and for variant calls using the SNPPET function. The DuplexCaller [12] was used to identify mutations supported by duplex reads in the common CRC driver genes *TP53, APC,*

SMAD2/4, FBXW7, PIK3CA and in the known resistance driver genes *KRAS, NRAS, BRAF, MAP2K1, EGFR, FGFR2, ERBB2, NF1*. Mutations supported by reads with this duplex configuration were inferred to come from double stranded DNA molecules.

4.5 Genome-wide DNA copy number analysis

BAM files from MBC-deduplication before removal of single-read consensus families were used to generate genome-wide DNA copy number profiles with CNVkit [27] (v0.8.1). CNVkit was run in non-batch mode with antitarget average size set to 30 kb. Data from healthy donor samples were used as the normal reference pooled dataset [12]. We then assessed each profile for amplifications of the known resistance driver genes *ERBB2, MET, FGFR2* and *NRAS/KRAS*.

4.6 Mutation clonality analysis

Absolute copy number data was estimated from the genome wide copy number profiles using the following assumptions: the lowest arm level loss corresponds to copy number 1, the modal chromosome number has a copy number between 2 and 4 and copy number states are approximately equally spaced. For the most conservative clonality estimate, we assumed that only one copy of resistance driver genes is mutated and that all copies of the tumour suppressor genes *TP53* and *APC* harbor the detected mutations as this leads to the highest clonality estimate for resistance drivers. The fraction of cancer cells sampled by ctDNA that harbored a resistance driver mutation at PD was calculated by first correcting VAFs for the influence of copy-number states and by then dividing the corrected VAF of resistance drivers by the corrected VAF of clonal TP53/APC mutations. Referencing the resistance driver mutations against clonal mutations corrects for differences in the admixed DNA from normal cells, which varies between patients. The clonality calculations were performed with formulas from [9].

4.7 Generation of *MAP2K1* transgenic DiFi cell lines and Western blot analysis

HEK293T cells were transfected with pHAGE-MAP2K1 and pHAGE-MAP2K1-P124S (Addgene plasmids #116757, #116427 and #116428 respectively, kindly gifted by Gordon Mills & Kenneth Scott) lentiviral constructs in combination with

packaging plasmids psPAX and pMD2.G (a gift from Didier Trono, Addgene #12260 and #12259 respectively) using TransIT-LT1 (Mirus). DiFi cells were transduced with the resultant viral supernatants in the presence of Polybrene (8 μg/mL). Transduced wildtype MAP2K1 overexpressing cells were selected using 5 μg/mL Puromycin. Mutant MAP2K1 cells (P124S) were selected by fluorescence-activating cell sorting for GFP-high cells on a Sony SH800.

Cells were treated for 4 hours with 6.25, 25, or 100 μg/mL cetuximab, or with vehicle control GCTS buffer. Total cell lysates were prepared with NP-40 buffer supplemented with protease and phosphatase inhibitors (Sigma). Western blotting used primary antibodies p-ERK (Cell Signalling Technologies #9101) and ERK (Cell Signalling Technologies #9102). HRP-conjugated anti-beta Tubulin antibody (Abcam #ab21058) was used as a loading control. Bands were detected using ECL Prime (GE Healthcare), and visualised on an Azure Biosystems C300 detection system.

5. Conclusion

Error corrected ctDNA-sequencing with a targeted panel allows the detection of broad genetic resistance mechanisms in CRCs treated with EGFR-Abs and chemotherapy. This may inform patient stratification to novel therapies and help to avoid ineffective treatment with EGFR-Abs. Our data furthermore shows a genetic resistance-gap after treatment with EGFR-Abs in combination with chemotherapy, indicating a need to investigate resistance mechanisms beyond the well described genetic point mutations and amplifications in receptor tyrosine kinases and RAS/RAF pathway members.

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