Heterogeneity of acquired resistance to anti-EGFR monoclonal antibodies in patients with metastatic colorectal cancer

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Abstract

Purpose
Even if RAS-BRAF wild-type and HER2/MET negative mCRC patients frequently respond to anti-EGFR monoclonal antibodies, acquired resistance almost invariably occurs. Mechanisms of resistance to EGFR blockade include the emergence of KRAS, NRAS and EGFR extracellular domain mutations as well as *HER2/MET* alterations. However, these findings derive from retrospective studies that analyzed one single resistance mechanism at a time; moreover, it is still unclear how molecular heterogeneity affects clonal evolution in patients. In this work, we aimed at extensively characterizing and correlating the molecular characteristics of tissue- and blood-based data in a prospective cohort of mCRC patients who received anti-EGFR antibodies.

Experimental design
Twenty-two *RAS-BRAF* wild-type, *HER2/MET* negative mCRC patients progressing on anti-EGFR therapy after initial response underwent re-biopsy. Next-generation sequencing and SISH/IHC analyses were performed both on archival tumors and post-progression samples. ctDNA molecular profiles were obtained in matched tissue-plasma samples.

Results
RAS mutations and HER2/MET amplification were the most frequently detected resistance mechanisms in both tissue and blood sample analysis. On the other hand, *BRAF* and EGFR ectodomain mutations were much rarer. Patients with acquired *MET* amplification showed worse PFS on anti-EGFRs. We detected both intra-lesion heterogeneity, as suggested by co-occurrence of different resistance mechanisms in the same sample, and inter-lesion heterogeneity. The combined analysis of tissue and blood (ctDNA) results highlights the complexity of clonal evolution triggered by EGFR blockade.

Conclusions
Our results indicate that it may be extremely challenging to target the complex landscape of molecular heterogeneity associated with emergence of resistance to targeted therapies in mCRC patients.

Statement of translational relevance

We extensively investigated the occurrence of mechanisms of secondary resistance to anti-EGFR agents in metastatic colorectal cancer patients. This work highlights how tissue-based and liquid biopsy can reveal the intralesion and interlesion molecular landscape that emerges in CRC patients treated with panitumumab and cetuximab. Our results indicate that a unique strategy cannot be readily defined to overcome secondary resistance to EGFR blockade, while pointing to the importance of early detection of resistant clones in individual patients to design personalized treatments.

Introduction

In the last decade, uncovering the molecular and genetic bases of metastatic colorectal cancer (mCRC) has enabled personalized treatments that improved patients' survival and limited administration of costly and potentially toxic treatments to selected groups of patients. In particular, anti-EGFR therapy is nowadays approved for subjects with *RAS* wild-type tumors (1-3). Additionally, *BRAF* mutations are commonly recognized as an additional biomarker of primary resistance to cetuximab and panitumumab (4). Even if still not validated, *HER2* and *MET* gene amplification are less frequent alterations that are also associated with treatment resistance (5, 6).

Despite the implementation of biomarkers in the daily clinical practice, patients who initially respond almost invariably develop secondary resistance. The genetic and molecular landscape of secondary resistance is heterogeneous and partially overlaps with that of primary resistance (7). Several resistance mechanisms have been described so far, both *in vitro* and in retrospective patients' cohorts: these include *KRAS*/*NRAS* (8, 9), *BRAF* (10, 11) and EGFR ectodomain (9, 12, 13) mutations, as well as *KRAS* (8), *HER2* and *MET* (5, 6, 14) amplifications.

However, the relative contribution and clinical significance of these alterations is far from being exhaustive due to several reasons: invasiveness of biopsy procedures and ethical issues that limit the number of feasible re-biopsies at progression; uncertain clinical applicability of data obtained from re-biopsies and liquid biopsies; small and heterogeneous published series mostly investigating one resistance biomarker at a time; poor applicability of advanced technologies in the real life setting. In this scenario, a comprehensive characterization of acquired resistance mechanisms, their relative distribution and co-occurrence in the same tumor or patient will hopefully help to define effective strategies to overcome or delay acquired resistance to treatment. Analysis of plasma circulating tumor DNA (ctDNA), if

coupled with highly sensitive technologies, is able to detect mutant DNA alleles down to 0.01% (15) and has been proposed as a novel method for non-invasive detection of heterogeneous molecular alterations underlying the evolution of resistance in metastatic tumors (11, 16).

In this study, we extensively characterized the most common mechanisms of acquired resistance to anti-EGFR MoAbs in patients with mCRC, with the aim of appreciating their significance in a real life setting and correlating tissue- and blood-based data including allele frequency and molecular heterogeneity.

Materials and Methods

Patients population

Eligible patients had diagnosis of mCRC and received cetuximab- or panitumumab-based therapy until progressive disease (PD) at Fondazione IRCCS Istituto Nazionale dei Tumori (Milan) and Istituto Oncologico Veneto of Padua. Since the detection of tumor clones harbouring resistance mechanisms may significantly drop after treatment discontinuation (17), collection of tumor re-biopsy and blood samples was always performed within one month (30 days) from detection of PD.

Inclusion criteria were divided in:

1) Clinical. Acquired resistance to anti-EGFR MoAb, defined as radiographical documentation of PD after prior objective response according to RECIST 1.1 to single agent panitumumab or irinotecan plus cetuximab in clearly irinotecan-refractory disease. Reintroduction of anti-EGFR monotherapy following a prior line of chemotherapy plus anti-EGFRs (discontinued for reasons other than PD) was allowed only if achieving a second RECIST response. At the time of PD documentation, tumor re-biopsy was performed on the most accessible site of progressing metastasis. Radiological documentation of PD events preceding tumor re-biopsy

should have occurred within 12 weeks from last dose of anti-EGFR therapy. Progression-free survival (PFS) and overall survival (OS) were calculated from the date of start of anti-EGFR treatment to the date of PD or death (or last follow up), respectively. All patients signed a written informed consent to allow re-biopsy procedures. Their consent was expressed within an agreement to participate to an observational prospective cohort study (INT 117/15) as part of a wide screening program for ethically approved, institutional phase 1 trials.

2) Molecular. All archival tumor samples were *RAS* and *BRAF* wild-type as analyzed by means of Sanger sequencing and had to be re-analysed with the same techniques used for post-progression tumor samples, as detailed in the next section. Only patients with tumors negative for both for MET and HER2 by silver in-situ hybridization (SISH) and immunohistochemistry (IHC) were included. Pre-treatment amplified clones had to be below the cut-off of 5% to be considered negative. Next-generation sequencing (NGS) had to confirm *RAS* and *BRAF* wild-type status and mutated subclones had to be undetectable.

Tissue-based experimental analyses

On both archival and post-progression samples, we performed: 1) next generation sequencing of 50 genes' hotspot regions included in the Hotspot Cancer Panel v2 (Life Technologies) by using the Ion Torrent Personal Genome Machine platform (Life Technologies). 2) Dual color SISH and IHC both for MET and HER2. On post progression samples only, we used a second custom panel to analyze 26 amplicons (2,77 kb) corresponding to the EGFR extra-cellular region (exons from 1 to 14), as previously described (18). Detailed description of the experimental methods is described in the Supplementary Materials.

Liquid biopsies: plasma collection, ctDNA isolation and Droplet Digital PCR analysis

Liquid biopsies were collected as previously described (17). Briefly, 10 mL of whole blood were obtained in EDTA tubes. Plasma was separated within 5 hoursand stored at -80°C until ctDNA extraction by Maxwell RSC ccfDNA Plasma Kit with the automated Maxwell® RSC Instrument (Promega) according to the manufacturer's instructions.

Droplet Digital PCR (ddPCR) was performed as described previously (Siravegna et al., Nat Med 2015 and Arena et al., STM 2016). The results were reported as fractional abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles. using the QuantaSoft analysis software (Bio-Rad) ddPCR analysis of normal control (from cell lines) and no DNA template controls were always included. Samples with too low positive events were repeated at least twice in independent experiments to validate the obtained results.

Results

Patients characteristics

We analysed samples from 22 prospectively treated patients, who achieved benefit from an anti-EGFR monoclonal antibody, and then developed resistance. Their demographics and disease characteristics are listed in Table 1. All except one patients had left-sided tumors. Archival samples mostly came from the primary site (45.5%) or the liver (45.5%). Biopsies at disease progression were performed on liver (54.5%), lung (13.5%), primary tumor site (9%) or other metastatic sites (23% collectively). At the time of PD, all tissues were sampled by needle biopsy except for the case of pts 5, 14 and 15, where a whole metastatic lesion was surgically removed. Median time elapsed from PD to tumor re-biopsy was 20 (range 7-30) days.

Overall, 13 patients (59%) received panitumumab and 9 patients (41%) received cetuximab; half of the patients were treated with re-introduction of anti-EGFR monotherapy after previous discontinuation for reason other than PD.

Median PFS and OS were 8 and 15.6 months, respectively. No differences in PFS and OS were observed according to treatment status (re-introduction: yes vs no).

Genetic alterations associated with clinical resistance

Table 2 summarizes the genetic alterations found in tumor biopsies after progression on anti-EGFR moAbs therapy. Among 22 evaluated patients, *KRAS* mutations were found in 7 pts (2 Q61H, 1 Q61K, 2 G12R, 1 G13D, 1 G12V), *BRAF* mutations in 2 pts (both were V600E), *HER2* amplifications in 3 pts, and, finally, *MET* amplifications in 4 pts. A potentially new resistance mechanism was found in pt n. 15 (Table 2), with a mutation in *AKT1* gene, determining a D46N amino acid substitution, detected in 18% of analysed cells. All DNA alterations found in the same biopsy sample were mutually exclusive, except for the case of pt n.16, in which both *MET* amplifications and *KRAS* Q61H mutation were found. No *EGFR* ectodomain mutation was detected.

Heterogeneity of resistant cell populations in single metastases

In analysed biopsies, the putative resistance mechanism was detected in a highly variable percentage of analysed cells, independently from the specific mechanism or the detection technique (Table 2). In particular, median and average mutant allelic frequencies normalized for tumor cell content were 34% and 37.28%, respectively (range 6-97%). The heterogeneity score (HS), an estimate of the percentage of cells expressing a mutant *KRAS* version (19), varied in the range of 12-110, with a median HS of 68 and an average HS of 62. This means that, in some patients (e.g. pt 5), the mutant allele was present in only a minority of cells, while in pt 22 mutant *KRAS* was probably associated with gene amplification. The two cases

with acquired *BRAF* mutations also presented low HSs (14 and 28). Moreover, *HER2* amplification was found only in small cell fraction in pt 5 (Figure 1), while heterogeneous *MET* amplification was described in pt 15 (Figure 2); interestingly, these two cases with heterogeneous IHC staining pattern are among the three patients whose sampling consisted in complete metastasis removal and not needle biopsy, as previously explained. Finally, we found that patients with acquired *MET* amplification had a significantly shorter median PFS during anti-EGFRs as compared to those without (6.6 versus 10 months, respectively; p=0.014) (Supplementary Figure 1). On the other hand, neither *KRAS*/*BRAF* mutations nor *HER2* amplification were predictive of shorter or longer PFS (data not shown).

Circulating tumor DNA analysis

For 11 out of 22 patients included in our study, blood samples collected at the time of resistance were available and plasma ctDNA was successfully analysed by ddPCR. In particular, we looked for hotspot mutations in *KRAS*, *NRAS*, *BRAF* and *EGFR* ectodomain gene sequences, as well as *MET* and *HER2* copy number variation (CNV). We found 3 *KRAS* mutations, 1 *EGFR* ectodomain mutation, 1 concomitant *KRAS* mutation and *HER2* amplification, 1 concomitant *BRAF* mutation and *MET* amplification. Most of gene mutations were found to have a low fractional abundance (ranging between 0.11% and 34%; median and average 7.83% and 12.67%, respectively), suggesting the presence of the mutation in a small fraction of DNA-releasing tumor cells.

Comparison between post-progression tumor biopsies and ctDNA analysis

Mutational and CNV analysis on ctDNA samples and their comparison with tissue-based data are summarized in Table 3 and easily assessable in Figure 3. Among 11 blood samples available, ctDNA analysis was fully concordant with tissue biopsy analysis in 4 cases (pts n. 1,

7, 13, 22). Conversely, one patient with undetected resistance mechanisms in tissue biopsy had mutated *KRAS* and amplified *HER2* in ctDNA (pt n. 14), while three patients with detected resistance mechanisms on biopsies had no alterations in liquid biopsies (pts n. 4, 5, 17), and, finally, in 3 cases with positive tissue biopsies, different or additional molecular alterations were detected in ctDNA (pts n. 12, 16, 20). Of note, 3 *KRAS* mutations with the highest HSs on tissue biopsies were confirmed by ctDNA analysis with relatively high fractional abundance. On the other hand, two cases of *KRAS*- (pt n.5) and *BRAF*- (pt n.17) mutated tumors with low HS on tissue sample analysis were not confirmed by ctDNA analyses. Finally, *EGFR* (pt n.12) and *BRAF* (pt n.20) but not *KRAS* (pt n. 14) mutations detected only in ctDNA were found to have a low fractional abundance (0.11%, 3.6% and 21.5%, respectively). Collectively considered, these results suggest that mutations that are present in the tumor bulk of one biopsied lesion are usually detected with both methods, while alterations that are present in a minority of cells are more easily lost with either method.

Discussion

To the best of our knowledge, this is the first study that extensively analyzed mechanisms of secondary resistance to anti-EGFR moAbs both in tissue and liquid biopsy analyses in patients with mCRC. Since anti-EGFR moAbs are often combined with chemotherapy agents, it is difficult to discriminate between activity of chemotherapy and anti-EGFR agents in individual cases. To circumvent this problem and to optimize patients' selection on the basis of initial sensitivity to anti-EGFR therapy, we only included initially *RAS-BRAF* wild-type (20), *HER*2 and *MET* non-amplified patients with a radiologically documented objective response to anti-EGFR monotherapy (or in combination with irinotecan in clearly irinotecan-refractory disease). In our opinion, this combination of genetic and clinical criteria guarantees the best selection of patients previously benefiting from cetuximab/panitumumab sensitive tumors as

possible. Interestingly, all but one tumors were left-sided, thus indirectly confirming recent evidences from randomized trials suggesting a different efficacy of anti-EGFRs based on primary tumor location (21, 22).

We found that the onset of secondary resistance to cetuximab or panitumumab is often associated with molecular alterations that likely converge on MAPK pathway reactivation, with *KRAS* mutations and *MET* or *HER2* amplification being the most frequent, thus pointing out the potential clinical relevance of inhibiting the MAPK cascade in resistant tumors (10). However, *KRAS* activating mutations, the most frequent mechanism, have not been successfully targeted so far (23); whether inhibitors of downstream KRAS effectors, e.g. MEK or ERK1/2, can successfully target *KRAS*-mutated CRCs will be investigated in future studies (10). On the other hand, other emerging resistance mechanisms, including *BRAF* and *PIK3CA* mutations, as well as *MET* and *HER2* amplifications, may be targeted by already available drugs or drug combinations, such as MEK1/2 or BRAF inhibitors combined with anti-EGFR moAbs in *BRAF*-mutated tumors (10, 24), the PIK3CA inhibitor alpelisib, the ALK/MET dual inhibitor crizotinib (18), and the HER2-targeted dual block with trastuzumab and lapatinib or trastuzumab and pertuzumab (25, 26). The very low rate (1 of 22 patients) of mutations in PIK3CA/AKT/mTOR pathway indicates that, differently from metastatic melanoma developing acquired resistance to BRAF inhibitors (27), this pathway is a less crucial determinant of tumor resistance to cetuximab or panitumumab in mCRCs. Even if codon 46 *AKT1* mutations were already described as somatic variants (28, 29), their functional validation should be assessed by future preclinical studies. In this study we were able to detect *EGFR* ectodomain mutations in only 1 out of 11 ctDNA available samples, and also with low fractional abundance; this suggests that this resistance mechanism is rare overall, and occurs in a small proportion of tumor cells.

We also found that the emergence of *MET* amplification correlates with poorer PFS during anti-EGFR treatment. This mechanism was already shown for *RAS* mutations as compared to *EGFR* ectodomain mutations (30). We have also previously demonstrated that pre-existing *MET*-amplified sub-clones drive tumor progression; now, we suggest that the aggressiveness of such emerging clones may be associated with shorter benefit from anti-EGFR agents (14, 18).

In post-progression mutational analyses, we mostly observed a variable proportion, and rarely the totality, of sampled cells expressing one specific resistance mechanism. This was especially evident in the case of *KRAS*-mutated tumors, where a highly variable HS was calculated.The most intuitive interpretation to this finding is that different cell subpopulations within a single metastasis may have selected different resistance mechanisms, and we only detected those alterations that have been reported in the literature (intra-lesion heterogeneity). One alternative hypothesis is that non-mutated subpopulations consist of non-resistant cells in dynamical equilibrium with resistant ones. However, this latter interpretation is highly unlikely, because resistant cells rapidly replace sensitive cells in progressing lesions (7), while we found in some cases only a small proportion (< 10%) of cells harbouring one specific mechanism of resistance. Of note, the finding of heterogeneous *HER2* and *MET* amplification in two patients (pt 5 and 15, respectively) whose sampling at progression derived from surgical removal of their whole metastases instead of needle biopsy, may indicate that more accurate sampling can improve detection of small clusters of resistant cells that are embedded within the tumor bulk. In our opinion, these data coherently support the hypothesis of intra-lesion heterogeneity in resistant tumors (19), as also suggested by the finding of two resistance mechanisms in different cell proportions within the same biopsy specimen (pt. 16).

By a clinical perspective, it is currently unknown whether and how the accumulating evidence on mechanisms of acquired resistance to anti-EGFR moAbs will translate into palpable clinical benefit in mCRC patients. The evidence of intra-lesion and inter-lesion heterogeneity in resistant mechanisms would likely preclude the possibility to successfully target all resistant clones with a single targeted approach. However, case reports published so far suggest that, despite clonal heterogeneity, one mechanism may become prevalent in some patients, who could therefore benefit from targeted inhibition of the selected mechanism (18, 31). However, our results underline how hardly mCRC will replicate recent successes in the treatment of *EGFR*-mutated lung adenocarcinomas becoming resistant to first-generation TKIs, in which third generation TKIs targeting T790M-mutated EGFR are able to produce prolonged disease remissions or stabilizations (32, 33).

To our surprise, in this study we observed poor concordance between DNA analysis on tissue biopsy samples and ctDNA compared with previously reported data (17). In particular, in 3 out of 11 cases, high-sensitivity ddPCR analysis of ctDNA did not confirm results found in tissue DNA analysis. One possible explanation is that, compared with pre-treatment conditions (11, 17), tumors resistant to anti-EGFR blockade display significantly higher genetic heterogeneity, with several cell clones harboring different genetic alterations; this may cause the co-existence of several resistant clones in different metastatic lesions, with the chance to miss the opportunity to catch some of them with either tissue or liquid biopsy. Our data also suggest that results of genetic analyses may be deeply affected by the sampling method and/or DNA sequencing techniques employed; in some cases, high-sensitivity ddPCR analysis of ctDNA can help to identify alterations that are not revealed in tissue biopsies, due to their low fractional abundance (as is probably the case of pt. 12), or their emergence only in non-sampled lesions. On the other hand, genetic alterations found on tissue, but not liquid biopsies, may indicate heterogeneity of DNA release by different tumor cell sub-populations,

probably as a consequence of clone-related (clone genetics, apoptotic rate) and environment-related variables (e.g. tumor vascularization).

Therefore, the best way to comprehensively characterize acquired resistance is far from being fully established. Despite the limits related to procedure invasiveness and limited sampling, tissue biopsies are less influenced by tumor environment characterisrtics, and also provide quantitative information (adjustment of minor allele frequency according to tumor cellularity) on the prevalence of the resistance mechanism(s) within the bulk of tumor cells. On the other hand, it is well known that single lesion biopsies are often missing to capture tumor heterogeneity (31). In the future, combining tissue biopsy, ctDNA analysis and radiological imaging could help to fully characterize the heterogeneity of occurring resistance mechanisms, while also clarifying their relative contribution to the clinical resistance.

In this study, mechanisms of acquired resistance remain uncharacterized for the 23% of evaluated patients. However, due to the presence of intra- and inter-lesion heterogeneity, this percentage is likely to be an underestimation of the real number of tumor sub-populations with still uncharacterized resistance mechanisms. This should stimulate future research to identify novel mechanisms of secondary resistance to anti-EGFR MoAbs.

In conclusion, this study highlights MAPK reactivation as the most prominent mechanism driving secondary resistance to cetuximab and panitumumab in metastatic CRCs. The fact that different pathways can converge on MAPK cascade reactivation excludes upfront co-targeting of EGFR and kinases upstream of these pathways to prevent selection of resistant clones, because cells would rapidly evolve and/or select alternative escape mechanisms. However, early detection of emerging mechanisms of resistance through liquid biopsies may precociously suggest rational combinations of anti-EGFR moAbs with agents targeting the evolving resistance mechanism (16).

Figure legends

Figure 1: Acquired heterogeneous HER2 protein over-expression and *HER2* gene amplification (pt 5). Archival biopsy tissue **(A,B,C)** and post-treatment biopsy tissue **(D,E,F)** of pt5. Hematoxylin and eosin (H&E) staining of the archival biopsy tissue **(A)** and of the post-treatment tissue **(D)**. Immunohistochemical analysis of p185/HER2 protein (p185 IHC) shows absence of protein expression in the archival biopsy tissue **(B)** and heterogeneous immunostaining with few neoplastic glands displaying strong immunostaining in the post-treatment tissue **(E)**. Dual color bright field in situ hybridization (ISH) for *HER2* gene (gray dots) and the centromeric probe for chromosome 17 (Chr. 17) (red dots) demonstrates no gene amplification both in the archival tissue **(C)** and in most of the neoplastic cells of the post-progression biopsy with *HER2* gene copy number ranging from 1 to 3 (mean 1.9) and Chr.17 copy number ranging from 1 to 3 (mean 1.8) (**F**). In the post-progression biopsy, small clusters and multiple *HER2* copies (>6) were also present in <10% of neoplastic cells, thus featuring gene amplification in a small clone of neoplastic cells **(F, Inset)**. Images were taken using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-Fi1 camera equipped with control unit-DS-L2 (Nikon). Images were assembled using Adobe Photoshop 6 (Adobe Systems, San Jose, CA). Original magnification x200 (A-F), x 400(Inset)

Figure 2: Acquired heterogeneous *MET* gene amplification (pt 15). In this figure, post-treatment biopsy is shown for pt 11. Hematoxylin and eosin (H&E) staining of the biopsy specimen **(A)**. Immunohistochemical analysis by using an antibody recognizing the intracellular domain of MET showed heterogeneous immunostaining, with about 10% cells displaying strong membrane staining **(B)**. Low magnification **(C)** and higher magnification (**D)** of dual color bright field in situ hybridization (ISH) for *MET* gene (gray dots) and centromeric region of chromosome 7 (Chr. 7) (red dots) is shown. Most neoplastic cells

demonstrate no *MET* amplification with a mean of 4.4 *MET* copy number and a mean of 3.6 copy number of Chr.7; however, about 10% of neoplastic cells contain small and large clusters along with multiple (>6) *MET* copies, thus featuring *MET* gene amplification. Images were taken using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-Fi1 camera equipped with control unit-DS-L2 (Nikon). Images were assembled using Adobe Photoshop 6 (Adobe Systems, San Jose, CA). Original magnification x40 (A,B), x200 (C), x 600(D).

Figure 3: Comparison between tissue-based and liquid biopsy analyses in the study populations. As shown in the legend, point mutations are identified by red, amplifications by blue, negative results by gray. Concordance and discordance between the tissue re-biopsy and liquid biopsy are easily assessable.

Supplementary Figure 1: Patients with acquired *MET* amplification have shorter PFS as compared to those with MET-negative tumors. PFS duration expressed as month time units is shown for individual patients with acquired *MET* amplification (blue histograms) and MET-negative (red histograms) tumors (panel A). Kaplan Meier curves show shorter median PFS of *MET*-amplified (blue) as compared with MET-negative (red) patients; moreover, around 40% and 15% patients MET-negative (but none of *MET*-amplified subjects) were still progression-free at 10 and even 20 months, respectively (panel B).

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Table 1. Patients and disease characteristics

Main characteristics	Number = 22 (%)	
Age		
Median (Range), years	58	(35 – 77)
Gender		
Male	12	(54.5%)
Female	10	(45.5%)
Primary tumor location		
Right Colon	1	(4.5%)
Left Colon	11	(50%)
Rectum	10	(45.5%)
Archival tissue site		
Primary	10	(45.5%)
Liver	10	(45.5%)
Other	2	(9%)
Rebiopsy site		
Primary	2	(9%)
Liver	12	(54.5%)
Lung	3	(13.5%)
Other	5	(23%)
Anti-EGFR monoclonal antibody		
Cetuximab	9	(41%)
Panitumumab	13	(59%)
Anti-EGFR re-introduction		
No	11	(50%)
Yes	11	(50%)

Table 2. Summary of the investigated mechanisms of acquired resistance.

ID	Mutations detected by NGS						HER2 IHC	HER2 amplification*	MET IHC (H-score)	MET amplification *
	Tumor content	Acquired mutation	Mutant alleles	Mutant alleles normalized for tumor content	HS	Founder mutations (mutant alleles; normalized for tumor content; HS)				
1	30%	KRAS Q61H	11%	37%	74	TP53 R213Stop (27%; 90%; 180)	*2+*	*NO*	*200*	*NO*
2	-	-	-	-	-	-	***2+***	***YES***	*0*	*NO*
3	90%	BRAF V600E	13%	14%	28	TP53 R249W (60%; 67%, 134)	*1+*	*NO*	*0*	*NO*
4	-	-	-	-	-	-	*2+*	*NO*	***300***	***YES***
5	70%	KRAS Q61K	4%	6%	12	TP53 R248Q (56%; 80%; 160) SMAD4 (63%, 90%; 180)	***2+#***	***NO****	*180*	*NO*
6	50%	KRAS G12R	17%	34%	68	APC L1488Stop (19%; 38%; 76) TP53 G187Stop (37%; 74%, 148)	*ND*	*NO*	*NA*	*NO*
7	-	-	-	-	-	-	*1+*	*NO*	*180*	*NO*
8	-	-	-	-	-	-	***3+***	***YES***	*180*	*NO*
9	-	-	-	-	-	-	*2+*	*NO*	*120*	*NO*
10	-	-	-	-	-	-	*2+*	*NO*	*100*	*NO*
11	-	-	-	-	-	-	*2+*	*NO*	*120*	***YES***
12	-	-	-	-	-	-	***2+***	***YES***	*120*	*NO*
13	-	-	-	-	-	-	*2+*	*NO*	*NA*	*NO*
14	-	-	-	-	-	-	*2+*	*NO*	*40*	*NO*
15	80%	AKT1 D46N	18%	22%	44	TP53 R282W (14%;17%;34)	*2+*	*NO*	*30*	***NO°***
16	65%	KRAS Q61H	36%	55%	110	TP53 P278S (52%; 80%; 160) ATM P3050 (49%; 75%; 150)	*2+*	*NO*	***300***	***YES***
17	70%	BRAF V600E	5%	7%	14	TP53 L265P (40%; 57%; 114) TP53 E171G (24%; 34%; 68)	***2+#***	*NO*	***210***	*NO*
18	75%	KRAS G12R	9%	12%	24	TP53 D281E (58%; 77%; 154)	*1+*	*NO*	*120*	*NO*
19	30%	KRAS G13D	6%	20%	40	SMAD4 Q75STOP (20%; 66%; 132) GNAS R201H (15%; 50%; 100)	*ND*	*NO*	*0*	*NO*
20	-	-	-	-	-	-	*2+*	*NO*	***300***	***YES***
21	-	-	-	-	-	-	*1+*	*NO*	*180*	*NO*
22	40%	KRAS G12V	32%	53%	106	TP53 D281 (22%; 36%; 72)	*1+*	*NO*	*180*	*NO*

* Amplification status is assessed by silver in-situ hybridization
** Presence of <10% cells with *HER2* amplification;
# Presence of a small area (<10%) with 3+ expression
° Presence of <10% cells with *MET* amplification

List of Abbreviations: HS, heterogeneity score; NA, not available.

Table 3. Mutations detected in tissue-based analysis as compared to liquid biopsy in the 11 matched cases.

ID	Tissue-based analysis					Liquid biopsy analysis
	Tumor content	Acquired genetic alteration	Mutant alleles	Mutant alleles normalized for tumor content	HS	ctDNA target gene mutations (% fractional abundance) or amplifications (CNV)
1	30%	KRAS Q61H	11%	37%	74	KRAS Q61H (12%)
4	-	MET amplified	-	-	-	-
5	70%	KRAS Q61K	4%	6%	12	-
7	-	-	-	-	-	-
12	-	-	-	-	-	EGFR G465E (0.11%)
13	-	-	-	-	-	-
14	-	-	-	-	-	KRAS G12D (21.5%); HER2 (CNV 4)
16	65%	KRAS Q61H; MET amplified	36%	55%	110	KRAS Q61H (1.3%)
17	70%	BRAF V600E	5%	7%	14	-
20	-	MET amplified	-	-	-	BRAF V600E (3.66%); MET (CNV 9)
22	40%	KRAS G12V	32%	53%	106	KRAS G12V (37.5%)

* Amplification status is assessed by in-situ hybridization

Figure 1

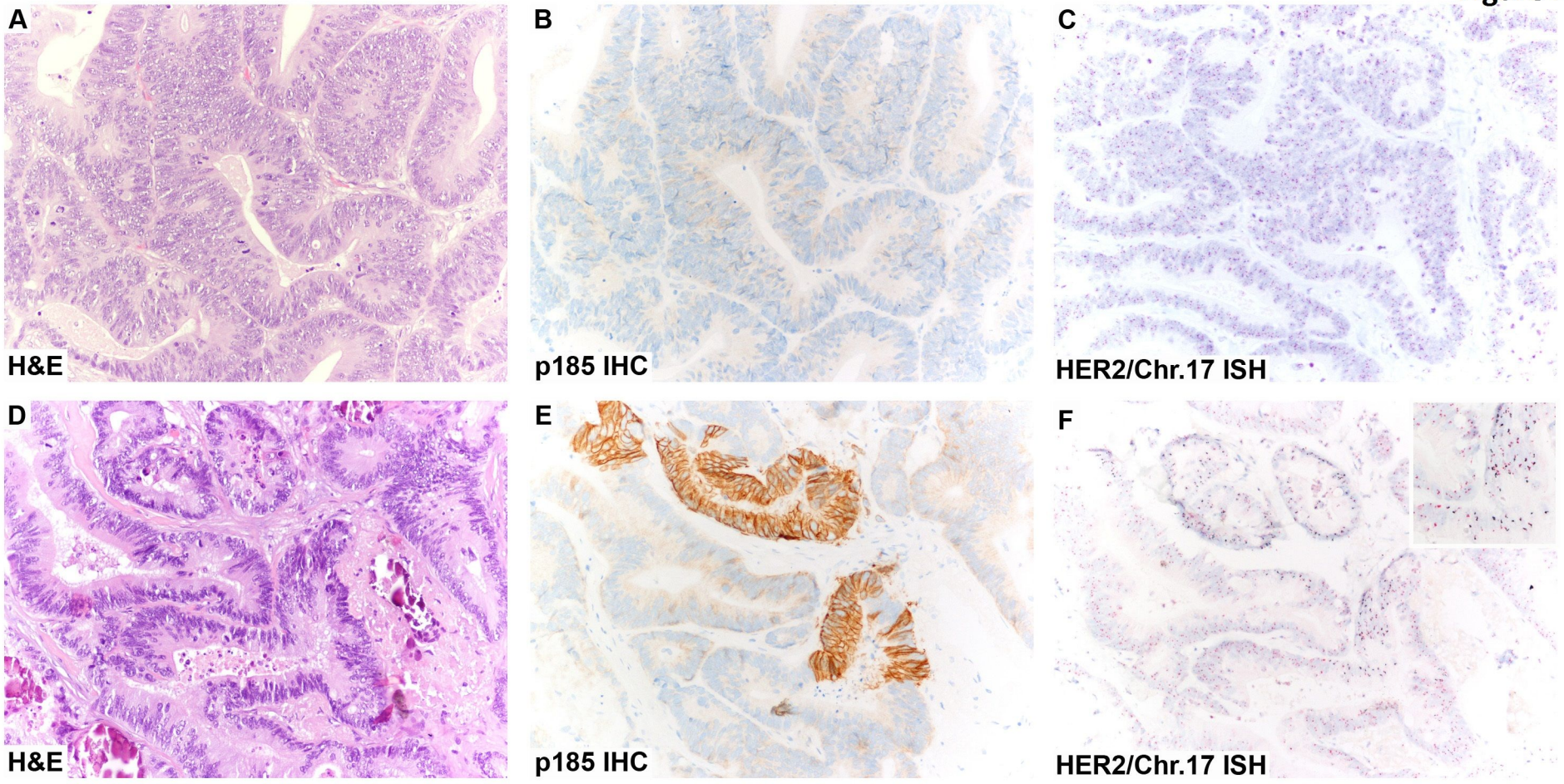
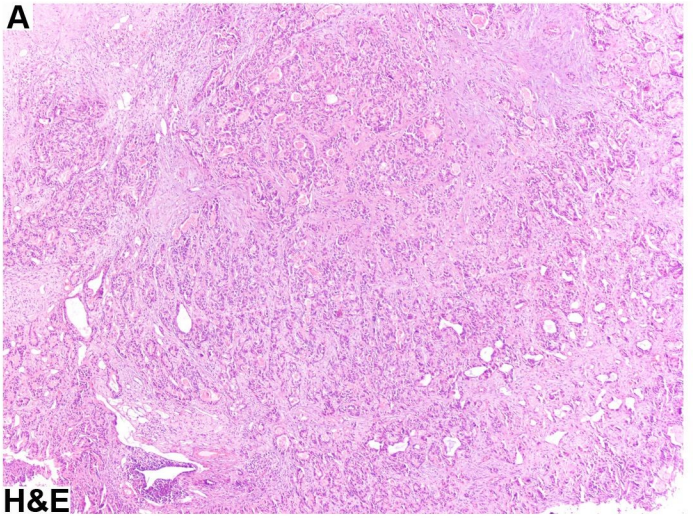
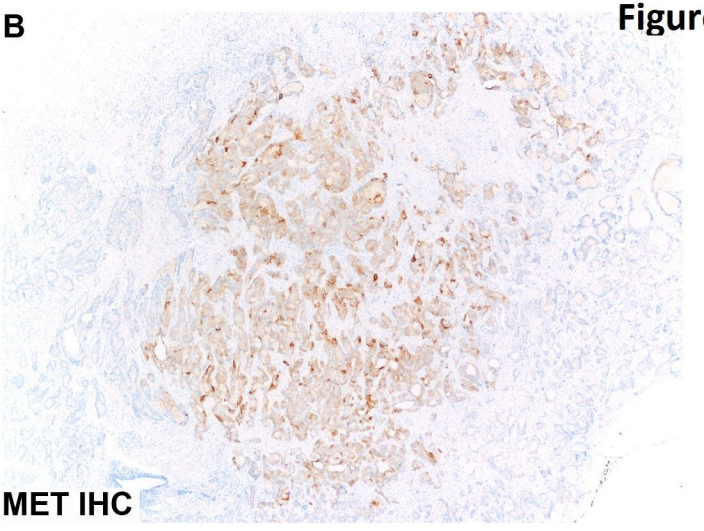
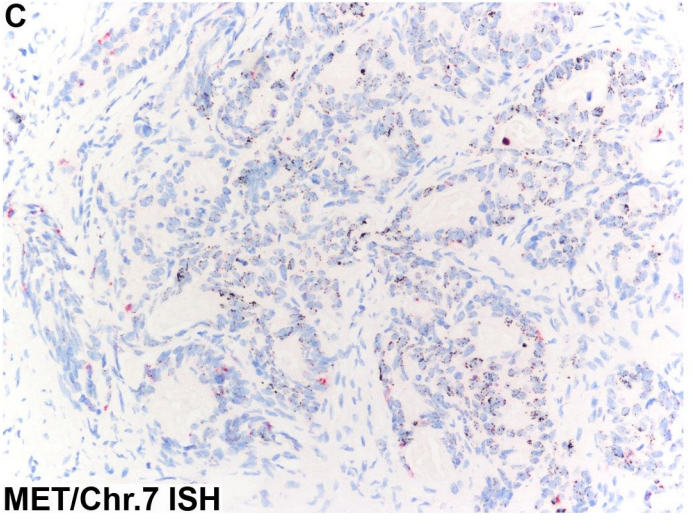


Figure 2







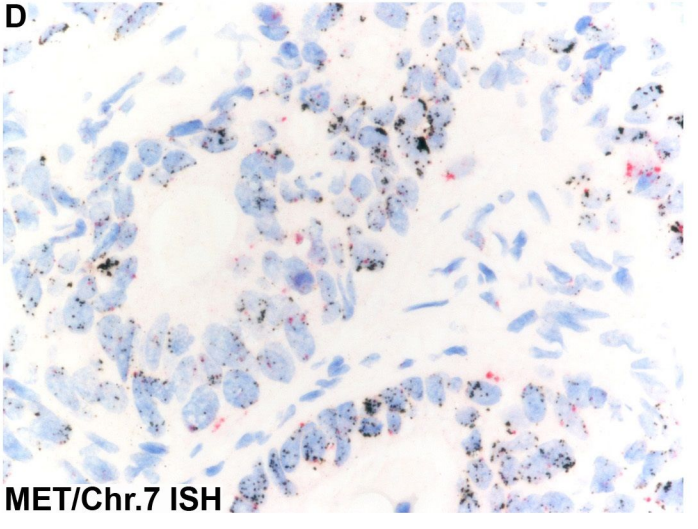
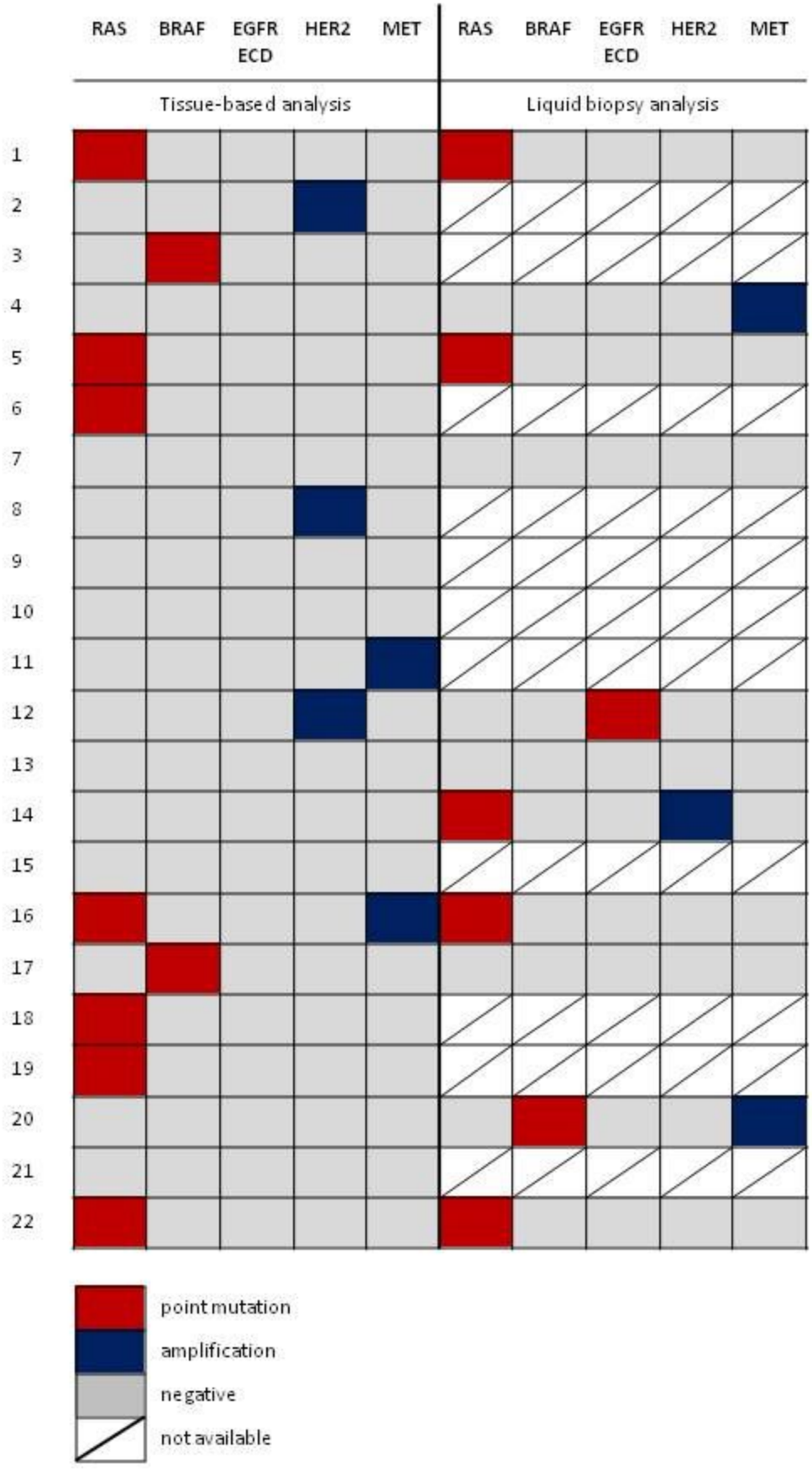


Figure 3


RAS
BRAF
EGFR ECD
HER2
MET
RAS
BRAF
EGFR ECD
HER2
MET
Tissue-based analysis
Liquid biopsy analysis
1
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21
22
point mutation
amplification
negative
not available