Genomic and Prognostic Heterogeneity Among RAS/BRAF^{V600E}/TP53 Co-Mutated Resectable Colorectal Liver Metastases

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Abstract

Background

Hepatic resection is potentially curative for patients with colorectal liver metastases, but the treatment benefit varies. *KRAS*/ *NRAS* (*RAS*)/ *TP53* co-mutations are associated with a poor prognosis after resection, but there is large variation in patient outcome within the mutation groups, and genetic testing is currently not used to determine the likely benefit from surgery. We have investigated the potential for improved prognostic stratification by combined biomarker analysis with DNA copy number aberrations (CNAs) in a tumor heterogeneity setting.

Methods

We determined the mutation status of *KRAS*, *NRAS*, *BRAF*, and *TP53* in 441 liver lesions from 171 patients treated by partial hepatectomy for metastatic colorectal cancer. CNAs were profiled in 232 tumors from 67 of the patients.

Results

Driver mutations in *KRAS, NRAS, BRAF*^{V600E}, and *TP53*, and high-level amplifications affecting cancer-critical genes such as *ERBB2* and *EGFR*, were predominantly homogeneous within patients. *RAS*/ *BRAF*^{V600E} and *TP53* co-mutations were associated with a poor patient outcome (hazard ratio, HR, 3.9, 95% confidence interval, CI, 1.3–11.1, $p = 0.012$) in multivariable analyses with clinicopathological variables. The genome-wide CNA burden and intra-patient inter-metastatic CNA heterogeneity varied within both the mutated and the wild-type groups. Combined prognostic analyses of *RAS*/ *BRAF*^{V600E}/ *TP53* mutations and CNAs, either as a high CNA burden or high inter-metastatic CNA heterogeneity, identified patients with a particularly poor outcome (co-mutation/high CNA burden: HR 2.7, 95% CI 1.2–5.9, $p = 0.013$; co-mutation/high CNA heterogeneity: HR 2.5, 95% CI 1.1–5.6, $p = 0.022$).

Conclusions

DNA copy number profiling identified genomic and prognostic heterogeneity among patients with resectable colorectal liver metastases with co-mutated *RAS*/ *BRAF*^{V600E}/ *TP53*.

Background

Approximately 30% of all colorectal cancer (CRC) patients develop metastases to the liver during their disease course, of whom 20% undergo hepatic resection as a potentially curable treatment (1, 2). In a Norwegian study, the five-year overall and disease free survival was 46% and 24%, respectively, after

partial hepatectomy (3), compared to a 5-year relative survival rate of 15–22% for patients with distant metastases from CRC overall (4). Around one third of the patients experience early recurrence following resection (3, 5, 6), and there are currently no strong markers for prediction of long-term benefit from surgery.

Mutations in *RAS* have consistently been associated with a poor prognosis among patients with resectable colorectal liver metastases (CRLM) (7–9), and it has been suggested that surgical treatment is less beneficial in patients with *RAS* mutations (10). However, the prognostic effect size is modest (11) and it was recently proposed that the effect is limited to tumors with co-occurring *TP53* mutations (12, 13), or co-occurring *TP53* and *SMAD4* mutations (14). $BRAF^{V600E}$ mutations have a stronger prognostic effect size, but the prognostic value is limited by the low prevalence of this marker among patients with resectable CRLM (15).

CRLM commonly presents with multiple distinct liver lesions. Cancer-critical genes with a high mutation prevalence in CRC generally have a homogenous mutation pattern across metastatic lesions from the same patient (16, 17), although treatment pressure may cause subclonal expansion, as illustrated by the emergence of resistant subclones with pre-existing or acquired *KRAS* mutations during anti-EGFR therapy (18, 19). More extensive mutation heterogeneity has been demonstrated in other protein-coding genes, both in intra-tumor and inter-tumor comparisons (20–22). We have previously shown that there is considerable intra-patient inter-metastatic heterogeneity also on the DNA copy number level (23). The clinical impact of such inter-metastatic molecular heterogeneity remains poorly defined (20, 21, 24), although our study suggested that a high degree of heterogeneity of DNA copy number aberrations (CNAs) are associated with a poor prognosis. We have previously also reported differential radiological responses to standard neoadjuvant treatment among metastatic lesions in a subgroup of approximately 10% of patients with resectable CRLM (25). How this phenotypic heterogeneity relates to molecular heterogeneity is currently not clear, but the poor survival rate of this patient subgroup after surgery highlights the potential clinical importance of inter-metastatic heterogeneity.

Here, we performed combined biomarker analyses in a tumor heterogeneity setting and in relation to outcome among patients with resectable CRLM. We investigated mutations in *KRAS, NRAS,* $BRAF^{V600E}$, and *TP53*, combined with the overall burden and inter-metastatic heterogeneity of CNAs.

Methods

Patient samples

The study included 460 liver metastases from 176 patients who underwent resection for CRLM at Oslo University Hospital, Oslo, Norway, between October 2013 and February 2018. Five patients were excluded due to mucinous tumor tissue, poor DNA quality, or suspicion of low tumor cell content. In total, 441 liver metastases from 171 patients were included for mutation analyses (Fig. 1), of which 102 patients had multiple lesions analyzed (median of 3 metastatic lesions per patient, range 2–9).

Fresh frozen tumor tissue samples (15–30 mg) were homogenized in liquid nitrogen and DNA was extracted using the AllPrep Universal DNA/RNA/miRNA protocol (Qiagen, Hilden, Germany). DNA quality and concentrations were assessed by NanoDrop 1000 spectrophotometer (version 3.7.1, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) and Qubit fluorometer (Thermo Fischer Scientific).

Mutation and microsatellite instability analyses

A total of 355 metastatic tumor samples from 103 patients have previously been analyzed for hotspot mutations in *BRAF* exon 15 and *KRAS* and *NRAS* exons 2–4 by Sanger sequencing (17). The remaining 86 tumor samples and 68 patients were analyzed in the present study.

All 441 tumor samples were also sequenced for all coding regions of *TP53* (exons 2–11). In summary, three singleplex PCR reactions were used to analyze *TP53* exons 2–4, 5–6 and 7–9, respectively, by amplifying 50 ng of DNA in a reaction mix containing 10x HotStar-buffer, dNTP, HotStar Taq polymerase (Qiagen), and the primers described in Supplementary Table 1. *TP53* exons 10 and 11 were analyzed in a separate multiplex PCR reaction by amplification of 50 ng of DNA using the 2x Multiplex PCR-kit (Qiagen). PCR products were purified using Illustra ExoProStar 1-step (GE Healthcare, Chicago, Illinois, U.S.A.), and the Applied Biosystems BigDye Terminator v1.1 Cycle Sequencing Kit and Applied Biosystems 3730 DNA Analyzer were used for sequencing (both Thermo Fisher Scientific). DNA from the blood of two healthy donors was used as controls. The results were analyzed using Applied Biosystems Sequencing Analysis software v5.3.1 and SeqScape software v2.5 (Thermo Fisher Scientific) and scored independently by two investigators. All mutations were validated in independent PCR reactions, and synonymous mutations were not called as mutations.

Five cases with intra-patient mutation heterogeneity of *TP53* among metastatic lesions were re-analyzed. Heterogeneous cases were first verified by a second Sanger sequencing, and two of the patients were subsequently re-analyzed also with ultra-deep targeted sequencing with the Illumina TruSight Tumor 15 gene panel as described in (17). High-sensitivity analyses disconfirmed *TP53* mutation heterogeneity in one patient. The other patient had inter-metastatic heterogeneity in the specific loci affected by *TP53* mutation, and displayed p.Asp184fs mutations in two lesions and p.Arg273His mutations in three lesions (all five lesions had the same *KRAS* mutation). The three patients for whom high-sensitivity data were not available were classified as mutated.

All tumors were analyzed for microsatellite instability (MSI) status using PCR-based marker analyses, either as previously described using BAT25/BAT26 (26), or using the five markers incorporated in the MSI Analysis System version 1.2 (Promega, Fitchburg, WI, USA). Uncertain cases after analyses of BAT25/BAT26 were re-analyzed with the MSI Analysis System. All patients had microsatellite stable (MSS) tumors except for one.

DNA copy number analyses

A total of 232 lesions from the first 67 patients with multiple metastases sampled were analyzed by genome-wide DNA copy number profiling using the Applied Biosystems CytoScanHD array (Thermo Fisher Scientific). The procedure was conducted according to the manufacturer's instructions, following the CytoScan Assay Manual Protocol. Resulting raw-intensity CEL-files were pre-processed with the R package rawcopy (v1.1) (27), and subsequently segmented by ASCAT (v2.5) (28), with penalty parameter set to 25 and chromosomes X and Y excluded. A primary interest was to estimate the level of CNA heterogeneity among samples from the same patient. This estimate is highly sensitive to poor data quality, and strict quality control was therefore performed on the segmented data by careful visual inspection of copy number profiles and Sunrise plots produced by ASCAT. Samples with non-aberrant profiles (no/few CNAs) or poor Sunrise plots were excluded, retaining 192 lesions from 64 patients for further analyses. Copy number gain and loss was called for segments with ≥ 1 or $\leq$-1 copies relative to the median genome-wide copy number estimated by ASCAT, respectively. For comparison with the data processing approach used in our previous study (23), the pre-processed data from rawcopy was additionally segmented by the PCF algorithm implemented in the R package copynumber (29) with the penalty parameter (gamma) set to 100.

To enable analyses across samples, the sample-wise segmented data were further split into their smallest genomic regions of overlap by computationally introducing breakpoints at every unique breakpoint occurring in any sample in the total dataset.

The COSMIC Cancer Gene Census version 86 (30) was used to define cancer-critical genes, (both Tier 1 and Tier 2 genes considered). Of the 719 genes, 672 were covered in the CNA data.

A sample-wise estimate of the overall CNA burden was calculated as the fraction of the genome (per cent of base pairs) with aberrant copy number. For patients with multiple lesions, the mean CNA burden was used for patient-wise analyses. Estimates of ploidy were derived from ASCAT.

Unpublished DNA copy number data was available for three matching primary tumors for comparison of amplification status in the metastases.

Estimation of intra-patient inter-metastatic copy number heterogeneity

High-quality DNA copy number data were available for at least two metastatic lesions from 48 patients (Fig. 1), including a total of 176 tumors and a median of 4 tumors per patient (range 2–8). These were analyzed for intra-patient inter-metastatic DNA copy number heterogeneity by three different approaches. First, the genome-wide matrix of estimated copy numbers was used to perform pairwise comparisons among metastatic lesions from each patient based on Euclidean distances, using the *dist* function implemented in the R stats package. To obtain one heterogeneity measure per patient, the mean Euclidean distance of all pairwise comparisons was calculated, in accordance with the approach used in our previous study (23). Second, the pairwise distance was calculated as in the first approach but using

Pearson correlation-based distance. Third, CNA heterogeneity was assessed by a gene-wise estimation (protein-coding genes from UCSC known genes) of the fraction of CNAs within a patient that were not common across the lesions, i.e. genes with aberrant copy number in one or more lesions but not in all. The heterogeneity-calling was more conservative with this approach, as only events exceeding the copy number gain/loss thresholds were considered heterogeneous, while genes consistently affected by gain (or loss) but with varying amplitudes were regarded as homogeneous CNA events.

The patient-wise CNA heterogeneity measure was categorized as high or low relative to the median across the patients.

For robustness, data segmented with the PCF algorithm was used to estimate copy number heterogeneity (distance-based) in the same manner as in our previous study (23), by calculating the average pair-wise Euclidean distance between DNA segments with a variance of > 0.03 among samples from each patient. The distance measured obtained from ASCAT and PCF showed good correlation (Spearman's rho 0.58, $p < 0.001$; Supplementary Fig. 1a).

Statistical analyses

Pairwise comparisons of variables between groups were done by non-parametric Wilcoxon rank-sum tests for continuous variables and with Fisher's exact test for categorical data, both implemented in the R stats package.

Survival analyses were performed with 5-year cancer-specific survival (5y-CSS) as the end point. Time to death from CRC was measured from start of treatment (either neoadjuvant systemic treatment or surgery) and deaths from other causes were censored (31). Only patients with MSS cancers and R0 or R1 status in the liver after resection were included in survival analyses (n = 165 of 171 patients in the full cohort, n = 62 of 64 patients in CNA burden analyses, n = 46 of 48 in CNA heterogeneity analyses). Kaplan-Meier estimates and log rank tests were used for comparisons of variables with only two groups, using the *survdiff* function in the R survival package. For comparisons of more than two groups, log rank tests for trend were performed using the *comp* function in the R survMisc package. All Kaplan-Meier plots were made with the *ggsurvplot* function in the R survminer package. Univariable and multivariable Cox regression analyses were performed with the *coxph* function in the R survival package.

Results

Concordant driver gene mutation profiles among multiple resected CRLM

Among resected CRLM from 171 patients, the patient-wise mutation prevalence was 42.7% (73/171) for *KRAS*, 4.7% (8/171) for *NRAS*, 1.8% (3/171) for $BRAF^{V600E}$ and 72.5% (124/171) for *TP53*. *KRAS*, *NRAS*

and *BRAF*^{V600E} mutations were mutually exclusive, while *RAS*/*BRAF*^{V600E} co-occurred with TP53 mutations in 31% of the patients (Fig. 2a). The mutation status of all four genes was homogeneous in all metastatic deposits analyzed from each patient when ultra-deep targeted sequencing was applied (three patients had unconfirmed heterogeneity; Fig. 2b). DNA copy number profiling indicated larger intra-patient inter-metastatic variation in these genes, and heterogenous DNA copy number status was found in 16/48 patients (33%) for *BRAF*, 24/48 patients (50%) for *KRAS*, 19/48 patients (40%) for *NRAS*, and 9/48 (19%) for *TP53* (Supplementary Fig. 1b). However, this was associated with a larger genome-wide level of CNA heterogeneity in the same patients (Supplementary Fig. 1c), indicating that these four genes were not specifically targeted.

None of the four genes had any high-level amplifications events (≤ 6 additional copies), but a genome-wide search identified high-level amplifications (≥ 15 additional copies) in CRLM from 22 (34%) of the 64 patients analyzed. Among cancer-critical genes (defined in the COSMIC Cancer Gene Census), recurrent high-level amplifications were found only of *ERBB2* in two patients, while *EGFR* and the cell cycle genes *CDK6*, *CCND2*, and *CCND3* were amplified in one patient each (Table 2). Additionally, the nominated target *TOX3* (32) was amplified in one patient. The amplification events were commonly concordant in intra-patient inter-metastatic comparisons, albeit with variation in the amplitude (Fig. 2b). Among patients with multiple metastases analyzed, 31% of high-level amplifications were homogenous, and an additional 38% of the amplifications had lower-amplitude gains (≥ 5 additional copies) in all other metastases from the same patient. Corresponding numbers for amplification events affecting cancer-critical genes were 50% and 25% (Supplementary Fig. 2). Notably, intra-patient concordance was also found for the clinically relevant target genes *ERBB2* and *EGFR*, including in comparison with the primary tumor of one patient with *ERBB2* amplification (9 additional copies in the primary and 17 and 18 additional copies in the two metastases). For the patient with two CRLM with *CCND2* amplifications (29 and 47 additional copies), the primary tumor had 32 additional copies of this gene. The patient with *CCND3* amplification in the range of 14–16 additional copies in all 7 metastases did not have a detectable *CCND3* amplification in the primary tumor.

Table 2
Inter-metastatic heterogeneity status for high-level amplifications of cancer-critical genes

Patient	Number of tumors analyzed	Region (hg19)	Cancer-critical genes in region	Copy number (range among tumors)*	Intra-patient inter-metastatic heterogeneity
1	7	chr6:39863162–42671542	*CCND3, TFEB*	14–16†	No (when also counting intermediate-level amplifications of 14 copies)
2	3	chr1:65183880–66527443 chr7:54576560–56118007 chr7:90792390–92573683 chr18:41497284–42716881	*JAK1* *EGFR* *AKAP9, CDK6* *SETBP1*	10–15 37–58 0–22 18–20	No (when also counting intermediate-level amplifications of 10–14 copies) No Yes No
3	2	chr12:4279446–4431071 chr16:40873444–53153010	*CCND2* *CYLD, TOX3*§	29–47‡ 0–16‡	No Yes
4	6	chr13:20528021–21570265 chr13:28302602–28662578	*ZMYM2* *CDX2, FLT3*	3–15 3–15	Yes Yes
5	5	chr17:37604254–37701703 chr17:37704051–38191836	*CDK12* *ERBB2*	16–41 41–55	No No

* Number of additional copies, relative to the estimated ploidy.

† Primary tumor: no amplification

‡ Primary tumor: 32 copies of *CCDN2* and a neutral copy number state for *CYLD* and *TOX3*.

§ Not a COSMIC gene.

| Primary tumor: 7 copies of *MLLT6*, 9 copies of LASP1, *CDK12*, *ERBB2*, 7 copies of *HLF* and 8 copies of *RNF43.*

Patient	Number of tumors analyzed	Region (hg19)	Cancer-critical genes in region	Copy number (range among tumors)*	Intra-patient inter-metastatic heterogeneity
6	2	chr17:36841569–37669141 chr17:37669142–37993556 chr17:53268056–53593625 chr17:56250122–57541594	*LASP1, MLLT6* *CDK12, ERBB2* *HLF* *RNF43*	12–18| 17–18| 14–18| 14–18|	No (when also counting intermediate-level amplifications of 12–14 copies) No No (when also counting intermediate-level amplifications of 14 copies) No (when also counting intermediate-level amplifications of 14 copies)
* Number of additional copies, relative to the estimated ploidy.					
† Primary tumor: no amplification					
‡ Primary tumor: 32 copies of *CCDN2* and a neutral copy number state for *CYLD* and *TOX3*.					
§ Not a COSMIC gene.					
| Primary tumor: 7 copies of *MLLT6*, 9 copies of LASP1, *CDK12*, *ERBB2*, 7 copies of *HLF* and 8 copies of *RNF43.*					

Frequent inter-metastatic DNA copy number heterogeneity on the genome-wide scale

The genome-wide CNA frequencies, summarized patient-wise, were in accordance with the well-known aberration profiles of CRC (among 192 lesions from 64 patients; Supplementary Fig. 3). Frequent copy number gains were found on chromosome arms 7p and q, 8q, 13q and 20q, and copy number losses on 1p, 4p and q, 8p, 17p and 18p and q. For the 48 patients with multiple metastases analyzed, inter-metastatic CNA heterogeneity was estimated by three different approaches (Methods) and with three different sets of input data of varying width of genomic coverage (across the whole genome, from protein coding genes, or from only the subset of 672 cancer-critical genes). The different estimates were strongly correlated, indicating robustness to both the approach (Spearman's rho ≥ 0.63, $p < 0.001$) and to the width of genomic coverage (Spearman's rho ≥ 0.93, $p < 0.001$; Supplementary Fig. 4). Further analyses were performed using the genome-wide Euclidean distance-derived heterogeneity measure, consistent with our previous study (23). There was a large variation among patients in the degree of inter-metastatic CNA

heterogeneity (Fig. 3a). This CNA heterogeneity was independent of the number of lesions analyzed per patient, the patient-wise median aberrant cell fraction and the *RAS*/*BRAF*^{V600E} mutation status, but was correlated with the patient-wise median ploidy state and ploidy range, and the *TP53* mutation status (Fig. 3b and c; Supplementary Table 2). The CNA heterogeneity score was also weakly correlated with the mean patient-wise CNA burden of the metastases (analyzed as the fraction of the genome with aberrant copy numbers; Spearman's rho 0.33, $p = 0.02$; Fig. 3c).

Co-mutated *RAS*/*BRAF*^{V600E} and *TP53* is associated with poor patient outcome

The 165 patients with MSS cancers that were treated with R0 or R1 hepatic resection had a median cancer-specific survival of 48 months and a 5y-CSS rate of 40%. The 139 patients with R0 or R1 status overall had a median cancer-specific survival of 50 months and a 5y-CSS rate of 44%. Several clinicopathological factors (Table 1) were associated with poor patient outcome in univariable Cox regression analysis, and gender, size of the largest metastasis, R-status in the liver and presence of extrahepatic disease remained significant in multivariable analyses (Supplementary Table 3).

Table 1
Clinico-pathological characteristics of all 171 patients and 48 patients with multiple metastases and associated CNA data

	Total patient series, n = 171		**Subset for copy number heterogeneity analyses, n = 48**	
Variable	**n (range)**	**%**	**n (range)**	**(%)**
Age at surgery, median (range)	66 (21–85)	-	67 (21–85)	-
Male sex	106	62	34	(71)
Primary tumor in right colon[a]	36	21	12	(25)
Positive nodal status primary	116[b]	68	28	(58)
Synchronous liver metastases[c]	134	78	39	(81)
Previous resection of CRLM	37	22	9	(19)
Previous chemotherapy	52	30	9	(19)
Chemotherapy for these CRLM	131	77	43	(90)
Targeted agents for these CRLM	47	27	17	(35)
Median (range) number of chemotherapy cycles	4 (1–41)	-	5 (1–41)	-
Median (range) size largest CRLM, mm[d]	27 (6-120)	-	29 (10–113)	-
Median (range) number of CRLM[d]	4 (1–23)	-	6 (1–20)	-
Median (range) number of analyzed CRLM	2 (1–9)	-	4 (2–8)	-
Laparoscopic procedure	39	23	3	(6)
Two-stage hepatectomy	33	19	18	(38)
Radiofrequency ablation	23	13	4	(8)
R-status liver				
R0-resection	71	42	14	(29)
R1-resection[e]	95	56	32	(67)
R2-resection[f]	5[g]	3	2[h]	(4)
Extrahepatic disease (%)	32	19	10	(21)

[a]Including the transverse colon

	Total patient series, n = 171	Subset for copy number heterogeneity analyses, n = 48
[b]Missing data for six patients		
[c]First liver metastases detected within 6 months of primary tumor diagnosis		
[d]On radiologic imaging before treatment		
[e]<1 mm margin or RFA treatment		
[f]Not completed second stage hepatectomy due to disease progression in observation period (n = 2) and missing lesions after neoadjuvant chemotherapy (n = 3)		
[g]Two patients with R2-resection of the liver also had extrahepatic disease		
[h]One patient with R2-resection of the liver also had extrahepatic disease		

RAS/*BRAF*^{V600E} mutations, but not *TP53* mutations, were associated with a poor 5y-CSS (*RAS/BRAF*^{V600E}: 32% for mutated versus 47% for wild-type, $p = 0.01$; *TP53*: 35% for mutated versus 55% for wild-type, $p = 0.1$; Fig. 4). Co-mutations of *RAS*/*BRAF*^{V600E} and *TP53* had a strong prognostic impact, with a 5y-CSS of 25%, compared to 46% for patients with *RAS* mutation only, 42% for *TP53* mutation only, and 71% in patients with wild-type status for all four genes ($p = 0.001$, test for trend, Fig. 4). Co-mutated *RAS*/*BRAF*^{V600E} and *TP53* was not significant compared to patients with *RAS* mutations only ($p = 0.2$). The prognostic role of co-mutations was not driven by patients with *BRAF*^{V600E} mutations, as the analyses remained significant upon exclusion of 3 patients with BRAF^{V600E} mutations (Supplementary Fig. 5a). Co-mutations of *RAS*/*BRAF*^{V600E} and *TP53* were enriched in patients with a right-sided primary tumor location and with extrahepatic metastases and depleted among patients with positive nodal status and those receiving neoadjuvant anti-EGFR or VEGF treatment (Supplementary Table 4). However, co-mutation was significant in multivariable analyses including clinicopathological factors (Supplementary Table 3).

Genome-wide CNA profiles have poor prognostic associations

Two measures of the CNA profiles of the CRLM were analyzed for prognostic associations among patients with MSS cancers and R0/R1 resection: the genome-wide CNA burden (the mean across lesions for patients with multiple CRLM analyzed) and the intra-patient inter-metastatic CNA heterogeneity estimate. Both these patient-wise CNA measures were categorized into a high and low group relative to the respective median in the patient series. High CNA heterogeneity or CNA burden was not overrepresented according to any of the clinical variables listed in Table 1. A high overall CNA burden was

significantly associated with a poor 5y-CSS rate, with survival rates of 15% and 44% in the high and low group, respectively ($p = 0.02$; Fig. 5). CNA burden was also significantly associated with a poor patient outcome when analyzed as a continuous variable (HR 1.03, 95% CI 1.01–1.05, $p = 0.009$). Furthermore, patients with high intra-patient inter-metastatic CNA heterogeneity also had a poorer survival rate than patients with a low heterogeneity, although not statistically significant in this smaller patient subgroup (5y-CSS of 23% and 37%, respectively, $p = 0.2$; Fig. 5). The combination of a high CNA burden and a high CNA heterogeneity was associated with a particularly poor patient outcome, and patients in this subgroup had a 5 year-CSS rate of 9%, compared to a 30% among patients with only one of the variables high and 50% among patients low for both CNA measures ($p = 0.02$, test for trend; Fig. 5). The median survival rates in the three groups were 25, 36 and 50 months, respectively.

Combined biomarker analyses suggest potential for stratification of the *RAS/BRAF*^{V600E}*/TP53* mutated subgroup by CNA profiles

Both CNA heterogeneity and CNA burden were significantly higher in patients with *TP53* mutated compared to wild-type tumors, but the CNA estimates were not associated with *RAS/BRAF*^{V600E} mutation status. Furthermore, there was a substantial variation in the CNA estimates within the mutational subgroups (Fig. 3b), motivating us to analyze the different prognostic biomarkers individually and combined. Within the *RAS/BRAF*^{V600E} mutated subgroup, the 5y CSS was 15% in patients with a high level of inter-metastatic CNA heterogeneity versus 42% in patients with low CNA heterogeneity ($p = 0.08$). Similarly, the 5y CSS was 0% in the *RAS/BRAF*^{V600E} mutated patients with a high CNA burden versus 44% in *RAS/BRAF*^{V600E} mutated patients with a low CNA burden ($p = 0.02$; Fig. 6a). Prognostic stratification of the *TP53* mutated subgroup by either of the CNA estimates was not statistically significant ($p \geq 0.2$; Supplementary Fig. 5b). The triple combination of co-mutation in *RAS/BRAF*^{V600E} and *TP53* and high inter-metastatic CNA heterogeneity was associated with a worse 5y-CSS compared with co-mutations/low heterogeneity and the remaining patients ($p = 0.02$ for analysis of trend among the three groups; Fig. 6b). Similar stratification of patients with co-mutations by the CNA burden showed a prognostic association also for patients with a triple combination of co-mutations and high CNA burden ($p = 0.01$, test for trend; Fig. 6b). Both associations were also supported by univariable Cox regression analyses (Supplementary Table 5). Similar results were found when excluding patients with extrahepatic metastases from the analyses (Supplementary Fig. 5c).

Discussion

Intra-patient molecular heterogeneity is anticipated to have clinical implications (33), and current evidence in metastatic CRC suggests that heterogeneity on the DNA copy number level is more widespread than heterogeneity of single nucleotide variants (SNVs) and small insertions/deletions (indels), at least in cancer critical genes (21, 23, 24). We have shown that mutations in *KRAS, NRAS,*

BRAF^{V600E} (17), and *TP53* are predominantly homogeneously present among multiple resected CRLM from each patient. The DNA copy number states of the four genes were more heterogeneous among metastases and correlated with the genome-wide inter-metastatic CNA heterogeneity, consistent with a lower selection pressure for these genes on the DNA copy number level than on the point mutation level. Furthermore, high-level amplifications targeting cancer-critical genes, including the therapeutic targets *ERBB2* and *EGFR*, were also typically homogeneously present within patients, both among multiple metastatic lesions and in the primary tumor. The timing of cancer-critical amplifications is poorly studied in CRC, and our results suggest that driver amplicons commonly arise before metastatic dissemination. In contrast, the level of genome-wide inter-metastatic DNA copy number heterogeneity beyond amplification events varied substantially among patients. There was no enrichment or depletion of cancer-related genes among genomic regions with heterogeneous DNA copy number, suggesting that CNA heterogeneity is a genome-wide and target-ignorant characteristic.

There is an urgent clinical need for markers that can identify CRLM patients who likely have limited benefit from surgery in combination with presently recommended perioperative oncological treatment. *BRAF*^{V600E} *and RAS* mutations are the molecular markers with best documented prognostic value, but their use in selection of patients for hepatectomy is currently not supported. *BRAF*^{V600E} has been shown to have the strongest prognostic effect size, but a low prevalence of only 3–5% among patients with resectable CRLM (15), and < 2% in this study. *RAS* mutations identify a larger patient subgroup, but have weaker prognostic value, which suggests molecular heterogeneity among patients with *RAS*-mutated cancers. In primary CRC, the prognostic value of *RAS* has been suggested to be limited to MSS cancers and to depend on the consensus molecular subtypes (34). In patients with resectable CRLM, the prognostic value may depend on co-occurring *TP53* mutations (12, 13) or *TP53*/*SMAD4* mutations (14). Here, we confirm the potential for improved prognostic stratification of CRLM after hepatic resection by *RAS*/*BRAF*^{V600E} and *TP53* co-mutations. Furthermore, we find that high inter-metastatic genomic heterogeneity confers poor outcome within the *RAS* mutated subgroup and show a potential for further prognostic stratification of the *RAS*/*BRAF*^{V600E} and *TP53* co-mutated subgroup by combined analyses with genome-wide CNA profiles. Although CNA burden and the level of CNA heterogeneity were independent of *RAS* mutation status, patients with *TP53*-mutated tumors had more extensive inter-metastatic CNA heterogeneity and a higher CNA burden than patients with wild-type tumors, suggesting a confounding prognostic effect. Loss of normal *TP53*-expression has previously been associated with tolerability to aneuploidy (35–41), and it is conceivable that *TP53* mutations are needed for a submissive state that allows extensive copy number heterogeneity to evolve. The CNA heterogeneity alone tended to be associated with poor outcome, while patients with higher than median CNA burden had significantly worse cancer-specific survival than patients with low CNA burden. The latter is in line with a recent pan-cancer study describing a significant association between a high CNA burden and poor disease-specific survival in patients with metastatic cancer (42). Our study was not powered to conclude on the independent prognostic value of CNA heterogeneity and *TP53* mutations in patients with *RAS*-mutated CRLM, although there was a significant trend for poorer patient survival in the *RAS*/*BRAF*^{V600E}/*TP53* co-mutated/high CNA heterogeneity group versus co-mutated/low heterogeneity versus remaining patients.

In accordance with the recent report from the Vauthey group (12), multivariable analysis with clinicopathological variables confirmed that co-mutation of *RAS*/*BRAF*^{V600E} and *TP53* was independently associated with poor patient survival.

It has been debated whether the association between residual disease and outcome may reflect underlying cancer biology, as mutated *RAS* is associated with both a positive resection margin and early development of lung metastases (8, 9, 43). However, excluding the patients with extra-hepatic metastases did not impact on the prognostic associations found in this study.

Conclusions

We have described genomic heterogeneity on the DNA copy number level in patients with resectable CRLM, also within patient subgroups defined by *RAS*/*BRAF*^{V600E} and *TP53* mutations. By combined biomarker analyses we confirm the superior prognostic value of *RAS*/*BRAF*^{V600E} and *TP53* co-mutations compared with either mutation alone. Furthermore, a high level of intra-patient inter-metastatic CNA heterogeneity or CNA burden may identify a subgroup of *RAS*/*BRAF*^{V600E}/*TP53*-mutated cancers associated with a particularly poor outcome.

Abbreviations

CNA – copy number aberrations; CRC – colorectal cancer; CRLM – colorectal liver metastases; 5y-CSS – five-year cancer-specific survival; MSI – microsatellite instable; MSS – microsatellite stable

Declarations

Ethics approval and consent to participate

All patients provided signed informed consents and the study was conducted in line with the Helsinki declaration with approval by the Norwegian Data Protection Authority and the Regional Committee for Medical and Health Research Ethics, South-Eastern Norway (ref no.: 1.2005.1629;2010/1805).

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article can be obtained from the authors upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors’ contributions

Study concept and design (KCGB, AS, AN, RAL); acquisition of data (all authors); analysis and interpretation of data (KCGB, THB, AS, AN, RAL); drafting of the manuscript (KCGB, THB, AS, RAL); critical revision and approval of the final manuscript (all authors); study supervision (AN, RAL)

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Figures

Figure 1

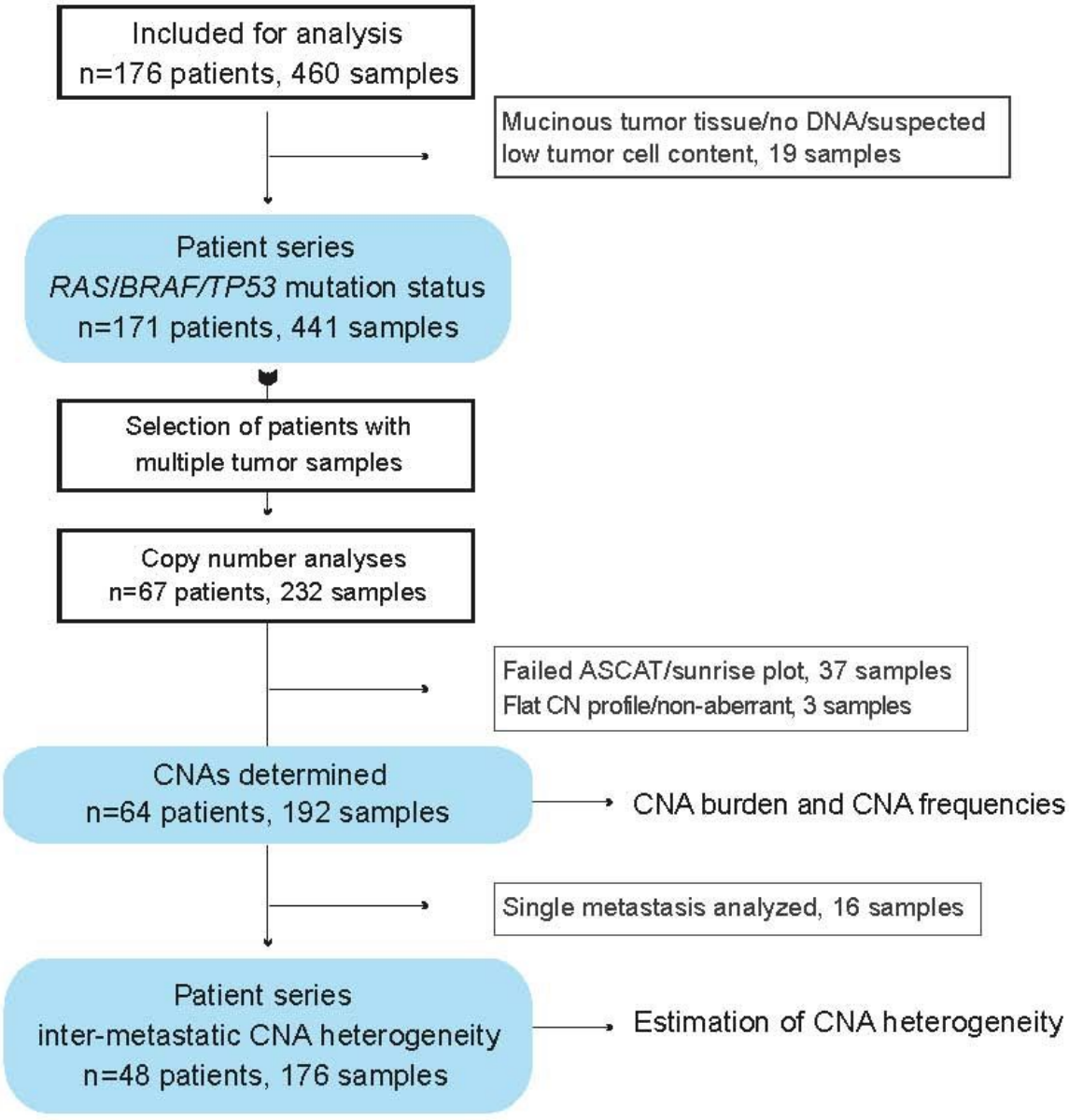

Figure 1

Overview of the included patients and samples in the study.

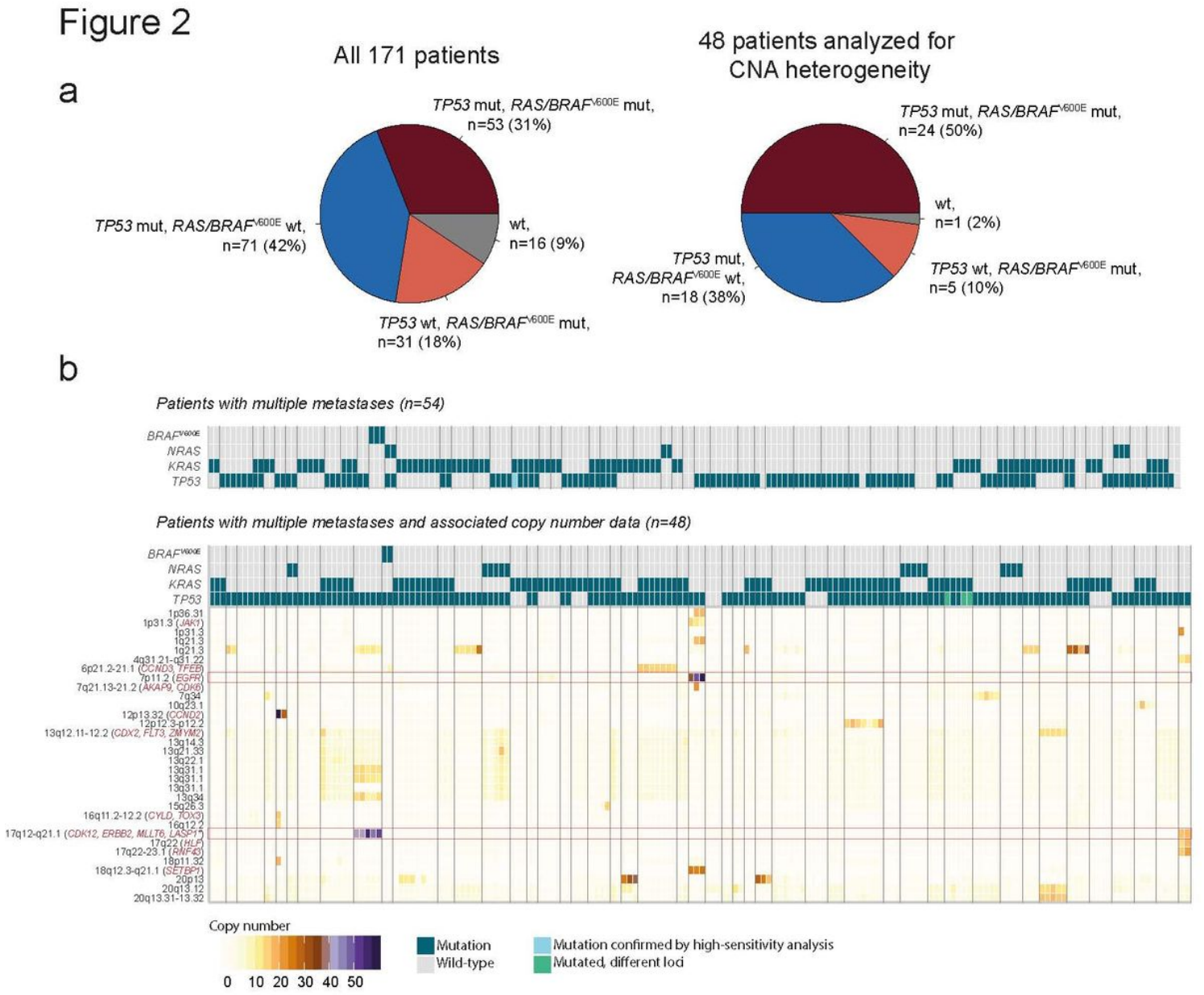

Figure 2

a) Prevalence of RAS/BRAFV600E mutation only, TP53 mutation only, and co-mutation of RAS/BRAFV600E/TP53 in the full cohort (n=171) and in the subset of patients with associated DNA copy number data from multiple lesions (n=48). b) The upper panel shows patients with multiple metastases that were analyzed by sequencing only (n=54 patients). The lower panel shows patients with multiple metastases analyzed for both mutations and CNAs (n=48 patients), and only lesions with good quality CNA data from the same resection was included. Vertical grey lines separate each patient. Cancer-critical genes are marked in red writing and the red horizontal boxes highlight the therapeutically relevant targets EGFR and ERBB2. The mutation status were the same in all metastatic deposits analyzed from each patient, with the exception of TP53 in four patients. One patient had TP53 mutations at two different loci among the lesions (pale green), and three patients had unavailable high-sensitivity sequencing data to rule out heterogeneity. Both mutations in the driver genes BRAF, NRAS, KRAS, and TP53, as well as high-

level amplifications (>15 additional copies), were predominantly homogenous within patients. *MLLT6 and LASP1: only amplified in the patient to the far right of the heatmap.

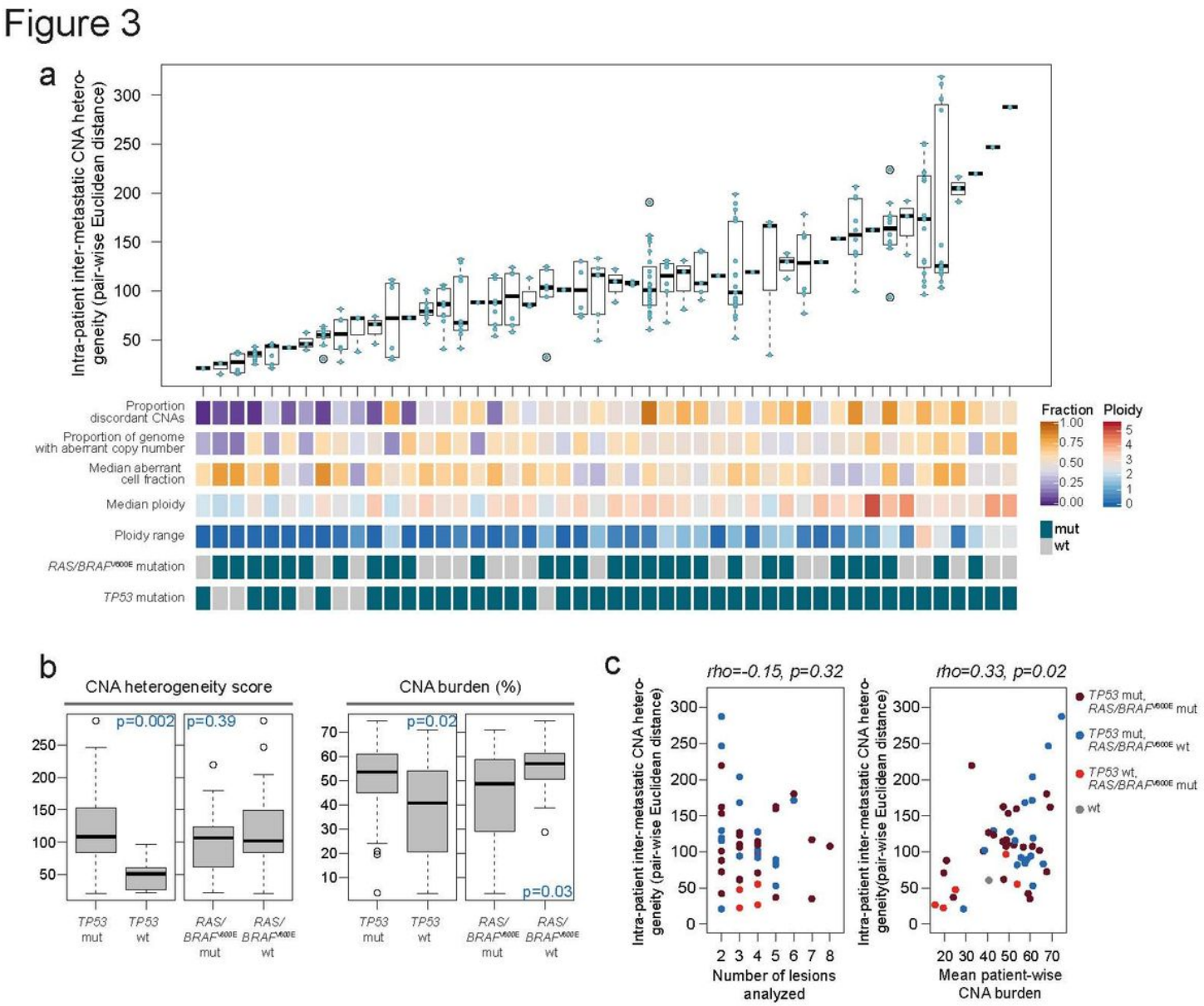

Figure 3

a) Genomic characteristics of 48 patients analyzed for DNA copy number heterogeneity. Top: Pair-wise Euclidean distance measures ranged between 21 to 319, and heterogeneity scores per patient (mean pair-wise distance measure per patient) ranged from 21-287 (median 104). Bottom: the bars indicate the fraction of CNAs found in one or more metastatic lesions but not all (discordant CNAs), the patient-wise average CNA burden (proportion of the genome with aberrant copy number), the patient-wise median and range of ploidy states among the metastases, and RAS/BRAFV600E and TP53 mutation status. b) CNA heterogeneity was significantly associated with TP53, but not RAS/BRAFV600E mutation status (n=42/n=6 TP53 mutated/wild-type; n=29/n=19 RAS/BRAFV600E mutated/wild-type). TP53 mutation was also associated with higher CNA burden, while RAS/BRAFV600E mutations were associated with

lower CNA burden (n=51/n=13 TP53 mutated/wild-type; n=42/n=22 RAS/BRAFV600E mutated/wild-type). The CNA estimates still varied within the mutational subgroups, with inter-quartile range between 27 and 65 for CNA heterogeneity (Euclidean distance) and 10-33 for CNA burden (%). c) CNA heterogeneity assessed as the mean Euclidean distance was not correlated with the number of lesions analyzed, and only weakly to the overall CNA burden.

Figure 4

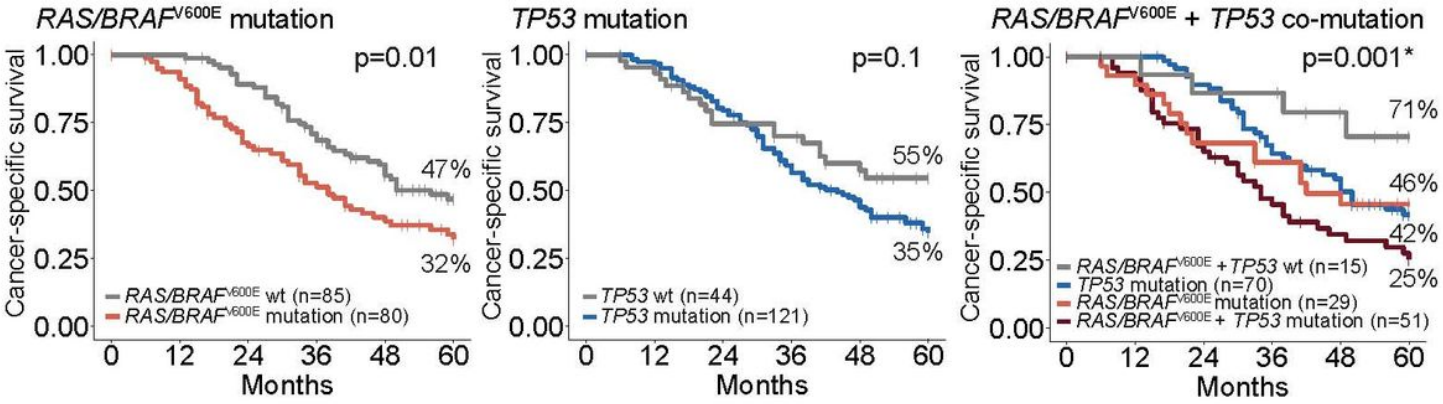

Figure 4

Five-year CSS according to mutation status. *Log rank test for trend. For pair-wise comparisons, RAS/BRAFV600E/TP35 co-mutation was associated with significantly worse survival than double wild-type (p=0.006) and TP53 mutation only (p=0.01), but not compared to RAS/BRAFV600E mutations only (p=0.2). Wt = wild-type.

Figure 5

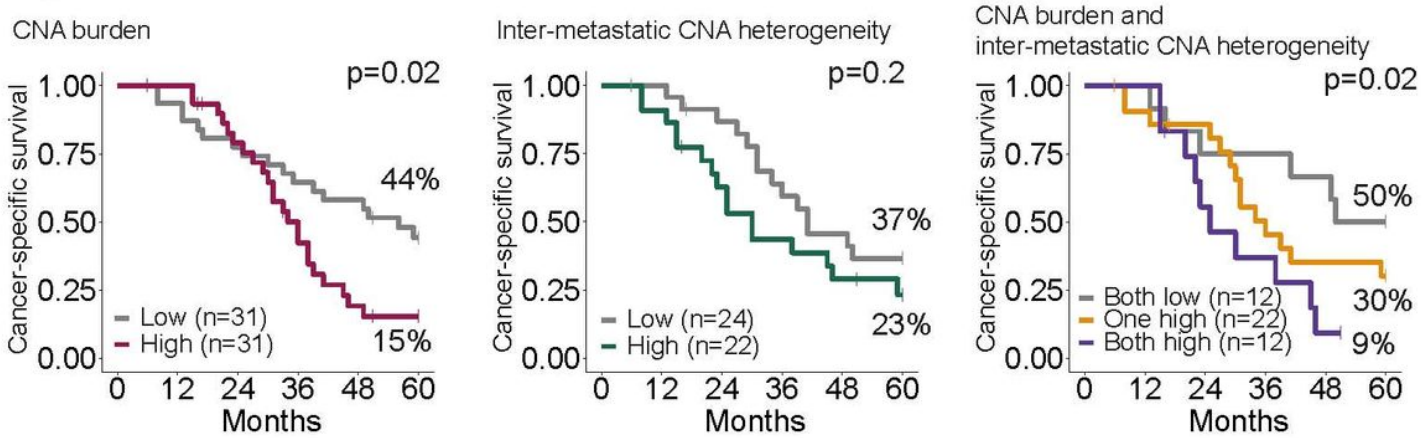

Figure 5

Five-year CSS according to CNA burden (left), CNA heterogeneity (middle) and both measures combined (right).

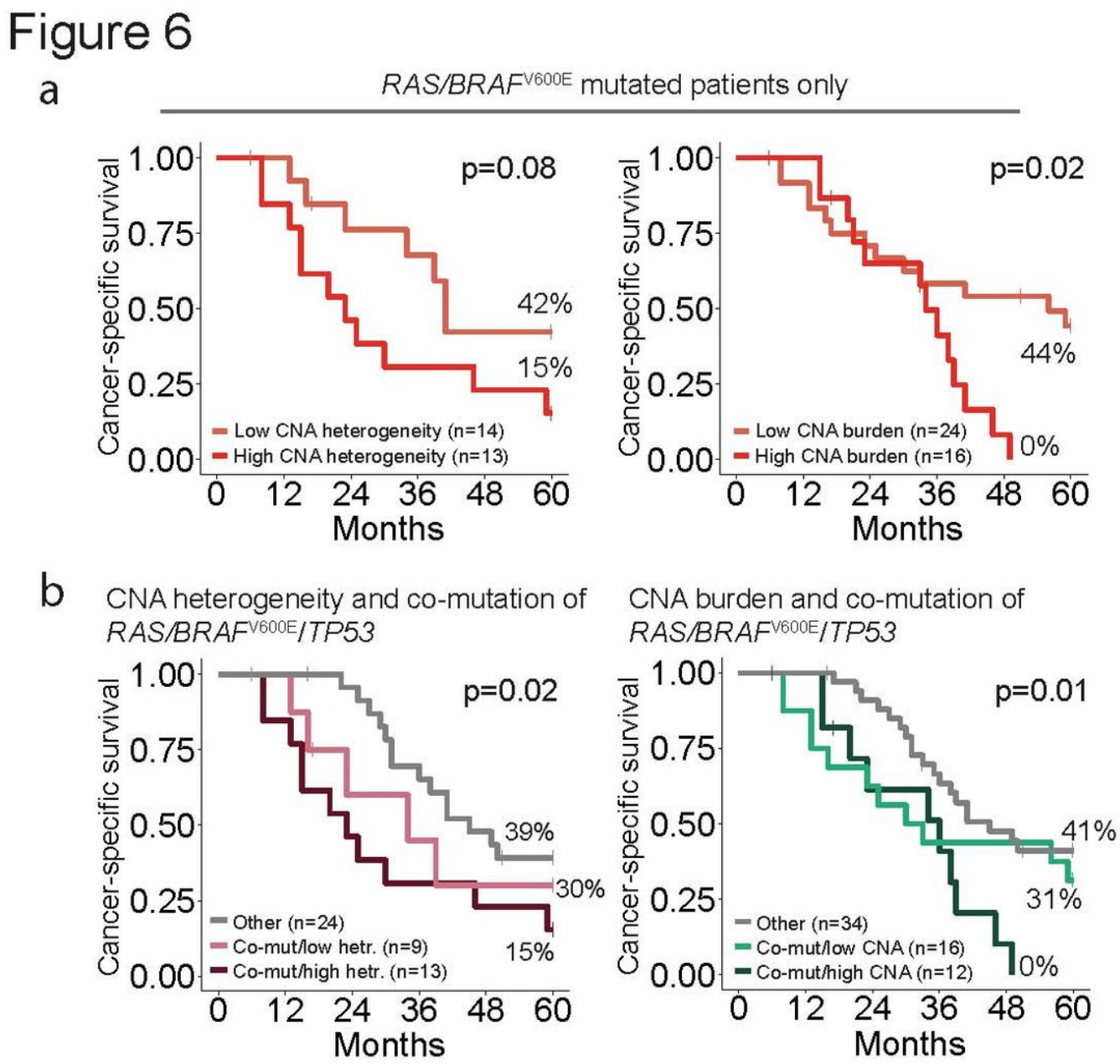

Figure 6

a) The RAS/BRAFV600E-mutated patient subgroup stratified by CNA heterogeneity (n= 27; left) and CNA burden (n=40; right). b) Patients with co-mutated RAS/BRAFV600E/TP53 stratified according to CNA heterogeneity (n=46; left) and CNA burden (n=62; right).

Supplementary Files

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