Mutation location on the *RAS* oncogene effects pathologic features and survival following resection of colorectal liver metastases

Running title: RAS mutation location and cancer biology

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PRECIS

We sought to determine if the location of driver mutations within the RAS oncogene impacts the biology of metastatic CRC. After stratifying patients by the exon location of mutation, we found dramatic differences in both survival and pathologic features.

ABSTRACT

Background

In the past three decades a better understanding of gene mutations and their role in carcinogenesis has led to improvement in our ability to treat patients with metastatic disease. We sought to determine if the location of a driver mutation within the affected gene impacts the biology of metastatic CRC.

Methods

DNA was collected from 165 randomly selected specimens of patients who underwent margin negative curative intent resection of colorectal liver metastases. Sequenom analysis and Sanger sequencing were used to evaluate mutations in K/NRAS, PIK3CA, BRAF and TP53.

Results

BRAF mutation was associated with early recurrence and death, while no impact of TP53 or PIK3CA mutation was identified. While K/NRAS mutation was associated with worse survival in this cohort, this difference was no longer evident when those receiving anti-EGFR therapy were excluded. When stratifying patients by the exon on which K/NRAS was mutated, there were dramatic differences in both survival and pathologic features. Exon 4 mutations were associated with large, solitary metastases occurring at long

disease free intervals as opposed to exon 3 mutations which presented with small, numerous lesions. Patients with exon 4 mutations recurred infrequently and had significantly longer survival when compared to wild type or other mutations.

Conclusions

Using this model of curative intent, R0 resection in patients at high risk of recurrence, we have been able to establish a link between mutation location within the K/NRAS gene and the biology of the metastatic CRC.

Key words: RAS mutation, colorectal metastases, BRAF mutation, hepatectomy, surgery

INTRODUCTION

Colorectal cancer represents the third most common malignancy in men and women in the Unites States with 135,000 new cases per year. Twenty percent of patients present with metastatic disease with many more developing distant spread during the course of their illness(1). The most common site of metastasis is the liver, which is present in nearly 80% of stage IV patients and the sole site of disease in 40%. Resection of colorectal liver metastases (CRLM) is associated with 5-year survival rates of 40 to 60%(2, 3) as well as occasional long-term cures. Five-year survival with systemic chemotherapy alone for stage IV disease approaches 10% in highly selected patients, requires chronic therapy and is typically non-curative (2, 4, 5). While risk scores based on clinical and pathologic parameters have been developed to predict outcomes after hepatic metastasectomy, these prognostic models tend not to impact clinical decision making and do not translate well across institutions(6). Therefore, novel and effective prognostic biomarkers are of obvious importance. At the root of the problem is a lack of

convincing data connecting the biologic drivers of carcinogenesis to the phenotype and survival after metastasectomy.

The mitogen activated protein kinase (MAPK) signaling pathway consists of a series of kinases which, when triggered by an extracellular signal, results in a downstream cascade influencing cellular proliferation, differentiation and survival. In the mid 1980's it was discovered that mutations in MAPK pathway genes, most notably *KRAS*, were integral to the initiation and progression of up to 50% of colorectal adenocarcinomas(7). Since that time, oncogenic mutations in other genes including *BRAF* (8) and *PIK3CA*(9) have been identified. Therapies targeting these pathways have been developed and RAS mutational status has emerged as a major predictor of response to anti-EGFR therapies(10, 11).

Controversy exists regarding the impact of MAPK mutations on recurrence and survival in patients with colorectal cancer. Previous studies investigating the association of MAPK mutations with the phenotype of metastases and impact on survival have been limited by three major factors; 1) inclusion of patients with a wide variety of tumor burden and stage, 2) failure to account for differences due to receipt of anti-EGFR therapy and 3) lack of analysis of less frequently mutated genes and exons such as *NRAS* and *KRAS* exons 3 and 4.

The aim of this study was to assess the impact of *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *TP53* mutations on recurrence and survival of patients undergoing curative intent complete resection of CRLM. This population of patients is at high risk of recurrence, but start with no macroscopic disease present reducing disease burden as a confounder.

METHODS

Patients

A prospectively maintained database of tissue samples obtained under an institutional review board-approved protocol was queried to identify patients who underwent resection of CRLM. All patients with formalin fixed paraffin embedded (FFPE) blocks and matched frozen tissue from resected colorectal liver metastases were selected for further analysis. Perioperative and survival data were collected by review of a prospectively maintained clinical database and supplemented by retrospective review of the medical record. Chemotherapy administration records were queried for administration of the anti-EGFR inhibitors cetuximab or panitumumab either pre or post-operatively. Only patients who underwent a margin negative (R0) curative intent hepatic resections were included. Pathology reports were used to capture tumor size and number and confirm margin status.

DNA extraction and sequencing

Hematoxylin and eosin stained slides prepared from FFPE tissue were reviewed by a GI pathologist (EV) to ensure adequate tumor content (>50%). Tumors with excessive necrosis were excluded from analysis. Genomic DNA was extracted from colorectal liver metastasis tissue using QIAmp DNA extraction kit (Qiagen, Valencia, CA) per manufacturer's protocol and subjected to whole genome amplification using the Repli-G Midi kit (Qiagen). The quality of whole genome-amplified DNA was verified by PCR reactions using two control amplicons. The mass array based iPLEX assay (Sequenom, San Diego, CA) was used to detect mutations in *KRAS* (codons

12,13,22,61,117 and 146), *NRAS* (codons 12,13,61), *BRAF* (codon 600) and *PIK3CA* (codons 345, 420, 542, 545, 546, 1043 and 1047), as previously described (12, 13). All mutations were confirmed by repeat iPLEX assay or Sanger sequencing. *TP53* mutations were assessed by routine Sanger sequencing.

Statistical Analysis

Nominal variables were evaluated using 2-tailed $\chi 2$ or Fisher's exact tests as appropriate. Continuous variables were assessed with univariate logistic regression for parametric values and Wilcoxon rank sum for nonparametric values. Kaplan-Meier survival curves were created to determine differences in time to recurrence and survival. Multivariate regression was performed using data that trended towards significance ($p<.10$ on univariate analysis and variables previously associated with the outcome. A p-value $<.05$ was considered significant.

RESULTS

Patients

After review of records of patients who underwent resection of colorectal cancer metastases between 1992 and 2009, we identified 211 patients who had resection of CRLM and had FFPE and frozen tissue samples available for further analysis. In order to investigate the impact of mutation status on survival, we excluded 41 patients with known residual disease at completion of operation to include only curative intent R0 resections. Of the remaining 170 patients, 5 others were excluded because they died of other causes (n=4), or died of unknown causes (n=1)(Figure 1).

Demographics and tumor characteristics

The mean age of the remaining 165 patients was 57.3 years (CI: 55.3-59.2) with a

slight predilection towards male gender (55%). This cohort consisted of a heavily pre-treated group with 72% receiving pre-operative chemotherapy and one-fifth having undergone a prior liver resection. Key pathologic features included a mean tumor size of 4.3cm (CI: 3.8-4.7) and a disease free interval of less than one year (Table 1).

Mutation status

Of the whole cohort of 165 patients, 50.6% had a single identified mutation, 30% had 2 or more mutations identified and 19.4% had no identifiable mutation. The most common mutation was in *TP53* (57.5%) followed by *K/NRAS* (43.0%; *KRAS* – n=65, *NRAS* – n=6). Other less frequent mutations included *PIK3CA* (12.1%) and *BRAF* (3.0%). The location of mutation in the *K/NRAS* gene was most often in exon 2 (codon 12 or 13 including G12D, G12S, G12V, G13C, G13D; 81.6% of RAS mutations), followed by exon 3 (codon 61 including Q61R and Q61H; 10.0% of RAS mutations) and exon 4 (codon 146 including A146T; 8.5% of RAS mutations)(Table 2).

Pathologic and demographic details by mutation status

Demographic details such as age and gender did not differ significantly between patients with different mutations or mutation location within the gene. There were also no differences in mutation pattern between patients who did and did not receive preoperative chemotherapy. There were no statistically significant differences in pathologic features of the metastases (number or size) or nodal status of the primary tumor by mutated gene (ie *WT* vs *K/NRAS* vs *PIK3CA* etc); however, location of the *K/NRAS* mutation within the gene was associated with varying pathologic features of the tumor. Exon 2 (n=58) mutant tumors had similar features to those without *K/NRAS* mutation with a mean size of 4.17 cm and an average of 2.4 tumors per resection. Those with mutations in exon 3 (n=7) had

a significantly greater number of tumors (mean=4.7 tumors), which tended to be smaller (mean=3.34 cm) and occurred at an earlier disease free interval (measured from date of liver resection) from resection of the primary (mean=3.4 months). Those with mutations in exon 4 (n=6) tended to have larger (mean=6.7 cm), solitary tumors that occurred after a longer disease-free intervals (mean=24.7 months) (Figure 2).

Overall and recurrence free survival by mutation status

At a median follow-up of 45 months, 49% of patients were dead of disease, 20% alive with active disease and 31% alive with no evidence of disease. The 3 and 5 year disease specific survival (DSS) for the whole cohort was 69% and 48%, respectively and the 3 and 5 year recurrence free survival was 30% and 26%, respectively. There were no differences in 5 year DSS in patients with *TP53* and *PIK3CA* mutations (5 year DSS of 47% and 50%, respectively) when compared to those without mutation (Figure 3 a,b). *BRAF* mutant patients had a significantly shorter 5 year DSS at 20% (Figure 3c). *K/NRAS* mutant patients had worse 3 and 5 year DSS compared to those without mutation (60 vs 76% and 38% vs 54%, respectively; $p<0.05$) (Figure 4a). In order to account for the potential confounding effect of anti-EGFR therapies, we then excluded patients that had received anti-EFGR treatment in either the *preoperative or post-operative periods*. Among the whole cohort of 165 patients, 44 received either cetuximab or panitumumab including 14 patients with and 30 patients without *K/NRAS* mutations. When these patients were excluded from analysis, the 3 and 5-year DSS of *K/NRAS* patients were no different than patients without mutation (Figure 4c).

We next looked at the prognostic impact of different locations of *K/NRAS* mutations within the gene. The 6 patients with mutations in exon 4 had a significantly

better 5-year DSS (83%) than those with mutations in exon 2 (35%) or no *K/NRAS* mutation (54%) ($p<0.05$). There was only one death among these 6 patients at 35 months; the other 5 being alive at 47, 72, 165, 183 and 229 months. The 7 patients with mutations in exon 3 had the worst outcomes with no patients surviving 5 years (Figure 5a). Exclusion of patients receiving anti-EGFR therapy had no impact on these results. A similar pattern was seen with recurrence free survival with only one exon 4 mutant patient recurring postoperatively compared to all of those with exon 3 mutations. The median time to recurrence for patients with exon 3 mutations was 12 months while those with no mutation or the more common exon 2 mutations recurred at a median of time of 20 and 18 months, respectively (Figure 5b).

To exclude potential confounding, multivariate analysis was performed including variables associated with recurrence free survival on univariate analysis as well as those previously identified as potential confounders (tumor size, number, disease free interval, nodal status of the primary tumor, prior chemotherapy and preoperative CEA level). Multivariate analysis identified only size of the metastasis and driver mutation as predictors of 5-year recurrence free survival with a risk ratio of 7.73 (CI 1.3-154;p=0.02) and 0.33 (CI .15-.88;p=0.03) for RAS exon 4 and exon 3 mutations when compared to no RAS mutation, respectively.

Patterns of recurrence

The gene and location of mutations were associated with the pattern of recurrence. *BRAF* mutant patients recurred in a multifocal pattern including the lung, liver and peritoneum. Patients with *K/NRAS* mutations recurred in the lung and liver with similar frequencies to wild type patients. This contradicts prior reports that suggested

K/NRAS patients more frequently recur with pulmonary metastases(14). When comparing recurrence patterns by location of *K/NRAS* mutation, those in exon 2 relapsed in a similar pattern to *K/NRAS* wild-type with an equal proportion of liver and lung lesions. Patients with exon 3 mutations had a more diffuse recurrence pattern with liver, lung and peritoneal disease (Figure 6).

DISCUSSION

Colorectal cancer remains a significant cause of cancer related mortality in the United States and worldwide. Patients often present with or develop metastatic disease, most commonly in the liver or lung. Metastasectomy for stage IV colon cancer has become an important tool in treatment of limited metastatic disease to the liver or lung and is associated with prolonged survival and occasionally, long-term cure(2, 15, 16). Despite advancements in pre-operative prognostic modeling, we are still unable to predict clinically relevant differences in outcome that can dictate therapeutic decision-making(6). As our insight into the biology of colorectal cancer improves, so too should our ability to prognosticate.

One of the most important advancements in our understanding of colon cancer biology was the discovery that a large proportion of tumors were driven by mutations located in the *MAPK* pathway, most notably *KRAS*(7). Since its discovery, the most clinically relevant finding related to *RAS* mutated tumors has been its ability to determine eligibility for treatment with anti-EGFR based therapy. More recently, *RAS* mutations have been studied for their potential to prognosticate recurrence and survival providing a link between genetic mutation and the biologic behavior.

Etienne-Grimaldi et al.(17) identified 93 stage IV colon cancer patients treated

with fluorouracil based chemotherapy and sequenced exon 2 of the *KRAS* gene. They identified mutations in 38.7% of patients and found no relationship between *RAS* mutation status and response or survival. Contrary to this, Span et al.(18) showed that survival in patients with all stages of colorectal cancer was significantly longer in patients with wild-type *KRAS* compared to *KRAS* mutants. Some of these discordant findings may relate to the way in which mutations in the *KRAS* gene are identified. Because a majority of mutations are found in exon 2, many researchers and clinical centers only sequence this portion of the gene. Recent data has shown that mutations in exon 3 and 4 as well as the related *NRAS* gene occur in an additional 10% of patients(13). As a result, some patients with *RAS* mutations are inadvertently grouped with non-mutated ones. Little is known about the biologic significance of varying mutation location within the *RAS* gene although in-vitro data suggests variable potential for activating downstream signaling which may result in differing biology(13). Data linking survival and phenotypic characteristics of tumors to more in-depth mutational analysis is lacking.

To study this, we chose a population of patients who had undergone complete resection of CRLM. These patients, as a group, have heterogeneous outcomes with both a high risk of recurrence as well as the potential for cure. By including only patients who have undergone a curative intent, margin negative resection, one removes the confounder of disease burden to get a clearer look at how driver mutations affect the biology of disease. A cohort similar to this has been the subject of two recent studies. Vauthey et al.(14) used a similar method of mutation capture and investigated the impact of *KRAS* mutation on survival and recurrence following hepatic metastasectomy. The authors found a significantly worse survival in patients with *KRAS* mutations compared to non-

mutated controls. One key limitation to this study was that the incidence of *KRAS* mutation was only 18% which is far lower than that reported by other groups. A second study by Karagkounis et al.(19) found similar results with a worse overall and recurrence free survival in patients with *KRAS* mutation compared to those without. This study also excluded patients who received preoperative anti-EGFR therapy and only analyzed exon 2 mutations, with no capture of *NRAS* or the exon 3 and 4 mutations. A recent report by Kemeny looked at both survival and recurrence pattern after hepatectomy by *KRAS* mutation and found an overall worse survival and a distinct pattern of recurrence. As in previous studies, non-exon 2 mutations were not routinely captured and this study included only patients who received hepatic arterial directed chemotherapy limiting the interpretation of results(20).

In the present study, an analysis of *MAPK* pathway mutations in 165 patients undergoing curative intent R0 resection of CRLM was undertaken to determine the prevalence and clinical significance of individual mutations. Sequenom mass spectrometry analysis was used to identify point mutations in exon 2, 3, and 4 of *KRAS*, exon 2 and 3 of *NRAS*, *BRAF* and *PIK3CA*. Clinical, pathologic and mutation data were available for 165 patients with a median follow-up of 45 months. There were no survival or pathologic differences in patients with *PIK3CA* or *TP53* mutations.

As has been previously reported(21, 22), patients with *BRAF* mutations were uncommon in this cohort and faired poorly. They tended to recur early and diffusely and none were disease free at 2 years (data not shown). The incidence of mutation was only 3% which is significantly lower than that reported by studies of primary tumors,(21) but in line with reports of metsastases(12, 14, 19, 23) highlighting the rarity in which these

patients present with resectable disease.

The incidence of *K/NRAS* mutation in our cohort was 43.0% which was higher than prior studies of hepatic metastasectomy(14, 19), but in line with other reports of metastatic lesions(17, 18). Using a more in-depth look at the *K/NRAS* gene, approximately 20% of these mutations occurred in less common locations on exons 3 and 4. This is important as these mutations are often excluded from traditional mutational analysis. When analyzing the whole cohort, *K/NRAS* mutation was associated with more frequent recurrence and worse disease specific survival(20) as we have previously reported. However, after eliminating patients who received anti-EGFR therapy before resection or at the time of recurrence, this difference no longer existed suggesting that the survival difference may reflect the efficacy of this therapy for *K/NRAS* non-mutants.

Based on in-vitro data, we hypothesized that the location of mutation within the *K/NRAS* gene may have an impact on tumor biology, recurrence and survival. Janakiraman et al(13) analyzed cell lines possessing *KRAS* mutations in either exon 2, 3 or 4. They found that mutations in exons 2 and 3 resulted in more robust downstream signaling and theoretically more malignant potential when compared to exon 4 mutations. To study this in-vivo, we grouped patients by site of *K/NRAS* mutation. In concert with the in-vitro findings, those with mutations in exon 4 had significantly fewer recurrences and an improved DSS compared to other mutation sites. Interestingly, patients with exon 3 mutations faired poorly, all recurring within 2 years and none surviving past 4 years. Multivariate regression confirmed these findings although small sample size limited the analysis as evidenced by the large confidence intervals. Pathologic features also differed by mutation location. Exon 4 mutated tumors tended to be large and solitary while those

with exon 3 mutations presented with numerous, smaller tumors.

As genetic sequencing becomes faster, more reliable and affordable, we are able to glean increasing amounts of information from resected tumors which can improve our ability to prognosticate and treat. This study reveals an even greater complexity than previously recognized where not only the identity of the driver mutation impacts the biology of disease but so to does the location of that mutation within the gene. While validation of these findings is needed prior to clinical applicability, it is reasonable to predict that in the near future, mutation status will be used in the same way as tumor size and multiplicity in guiding our decision to pursue increasingly aggressive metastasectomies.

Limitations of this study include its retrospective nature and single institution sample. We also focused only on hotspot mutations, which fails to take into account the many other mutations present in colon cancer. It is possible that unrecognized mutations might be acting in synergy with KRAS mutations to amplify the findings we observed. Some patients and tumors were excluded due to poor quality of the slides or excessive tumor necrosis. Finally, because many of these mutations occur infrequently, in-depth analysis was limited by the low number of patients in each group; particularly for patients with exon 3 and 4 *K/NRAS* mutated tumors.

In conclusion, we have demonstrated an association between tumor phenotype and specific gene mutations as well as location of the mutation within the gene. This serves as early data to suggest a possible new tool to help prognosticate outcomes following metastasectomy and aid in pre-operative patient selection. Further study including prospective collection of mutation status and analysis of larger populations is

needed.

FIGURE LEGENDS

Figure 1 – Flow chart outlining the selection and exclusion populations for the studied cohort.

Figure 2 – *K/NRAS* mutation location is associated with varying tumor phenotype: Patients with exon 4 mutations had larger tumors (a) and a longer disease free interval (c) compared to other mutation sites. Exon 3 mutation was associated with greater number of tumors (b).

Figure 3 – Kaplan-Meier curves showing overall survival for patients with or without a) *PIK3CA*, b) *TP53* and c) *BRAF* mutations.

Figure 4 – Overall survival by *K/NRAS* mutation: a) Kaplan-Meier curves demonstrate a difference in survival by *K/NRAS* mutation. b) Receipt of anti-EGFR therapy by mutation status. c) After exclusion of those receiving anti-EGFR therapy, there is no longer a difference in survival by *K/NRAS* status.

Figure 5– Overall survival (a) and recurrence free survival (b) by *K/NRAS* mutation location.

Figure 6 – Incidence of recurrence location by a) mutated gene and b) location of mutation on the *K/NRAS* gene of those patients who experienced relapse of disease.

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Table 1 – Patient characteristics and pathologic variables (n=165)

Characteristic	Value
Age (mean + CI)	57.3yrs (55.3-59.2)
Gender	55% male (n=91)
Synchronous disease	18% (n=30)
Mean disease free interval (mean + CI)	8.7mos (6.2-11.1)
Number of tumors (median + range)	2 (1-12)
Tumor size (mean + CI)	4.3 cm (3.8-4.7cm)
Pre-op CEA (mean + CI)	81.6 (33.4-129.8)
Pre-op chemo	91 (72%)
Previous liver resection	26 (19%)

Table 2 – Mutation data

Mutation site	Number (165)	Percent of total
No detected mutation	32	19.4%
K/NRAS	71	43.0%
Exon 2	58	35.1%
Exon 3	7	4.2%
Exon 4	6	3.6%
PIK3CA	20	12.1%
BRAF	5	3.0%
TP53	95	57.5%

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Figure 2

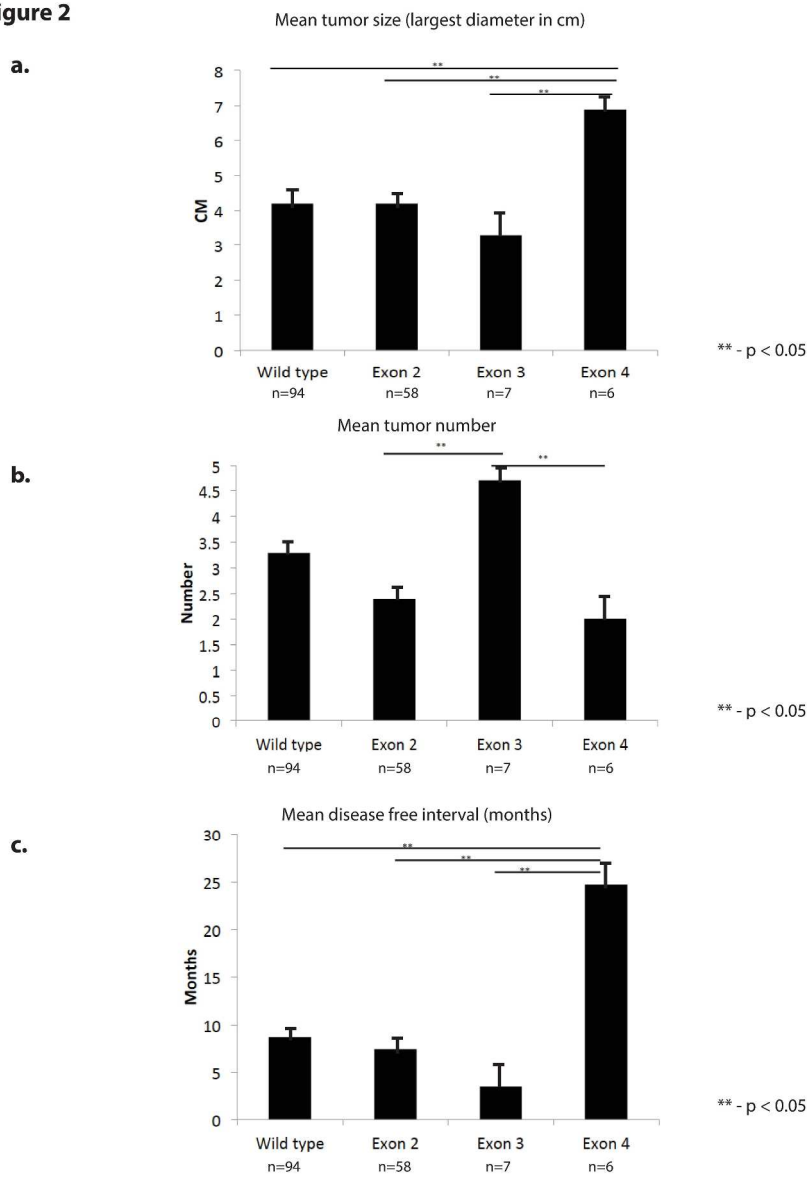

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Figure 3

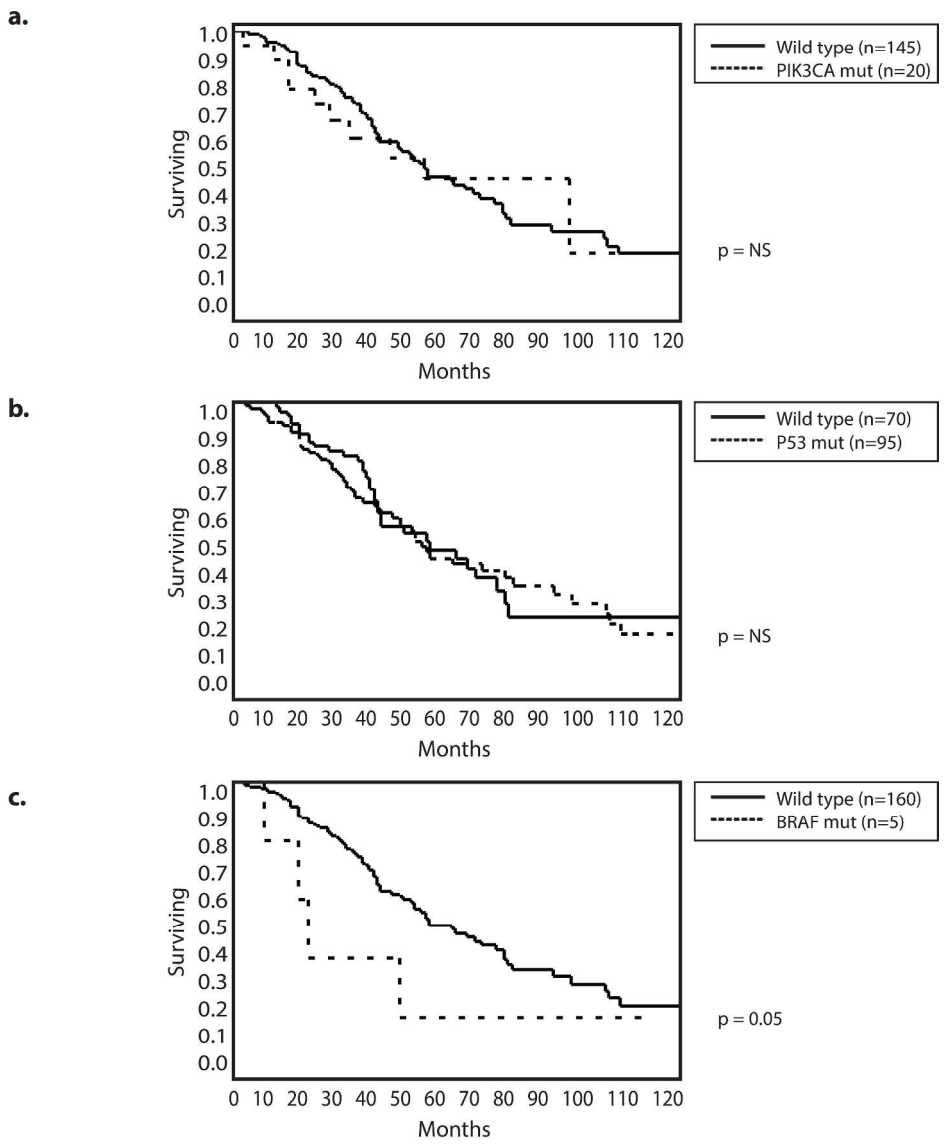

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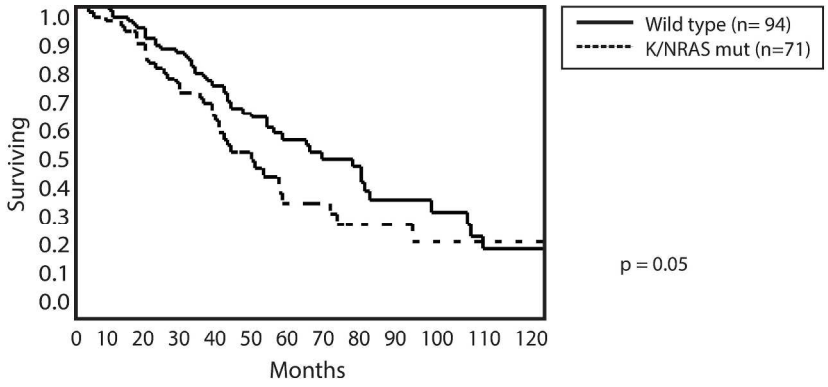
Figure 4

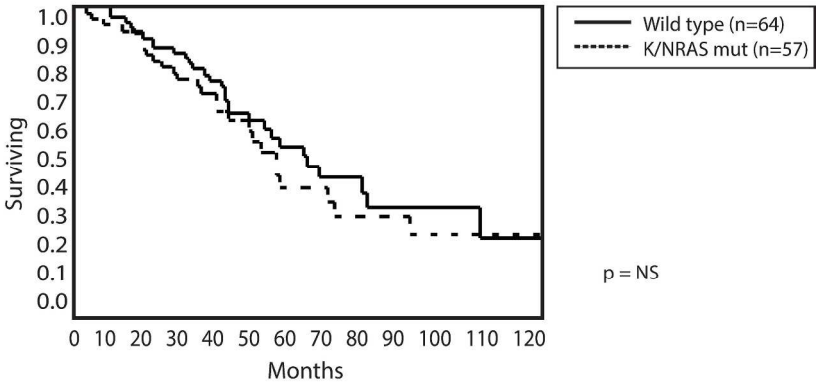
a.



b.

Mutation	Anti-EGFR	Percent
Wild type (n=94)	30	31.9%
K/NRAS mutant (n=71)	14	19.7%

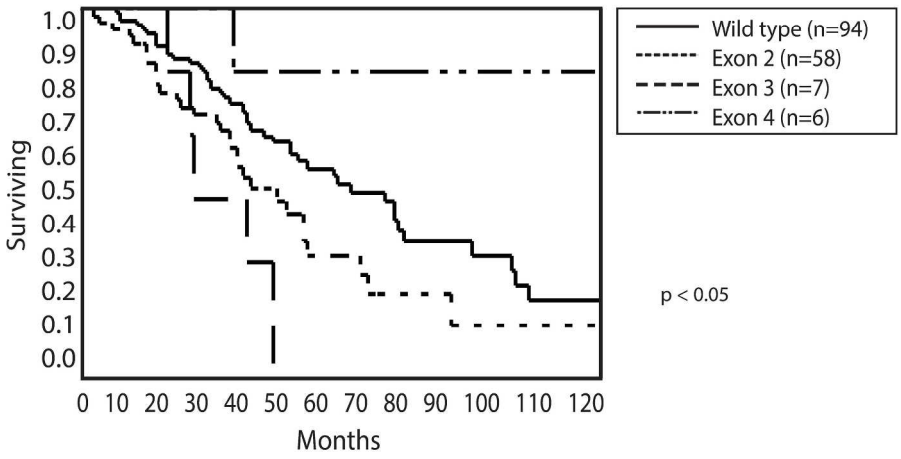
c.



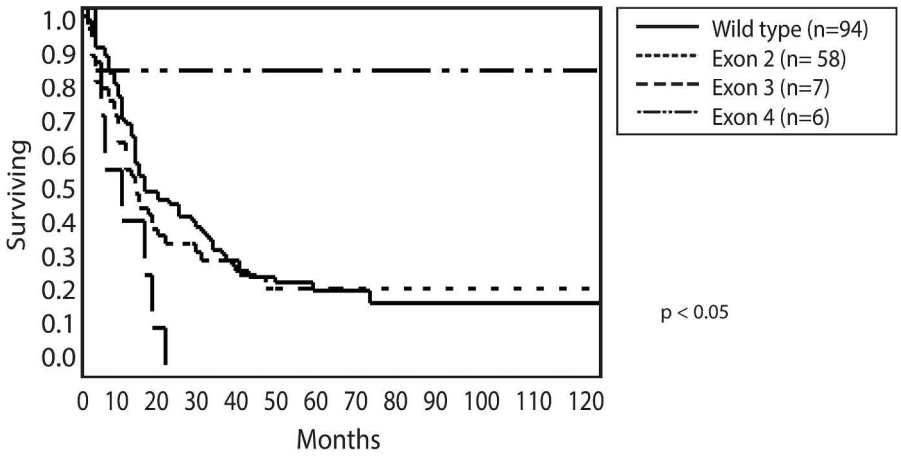
247x307mm (300 x 300 DPI)

Figure 5

a.

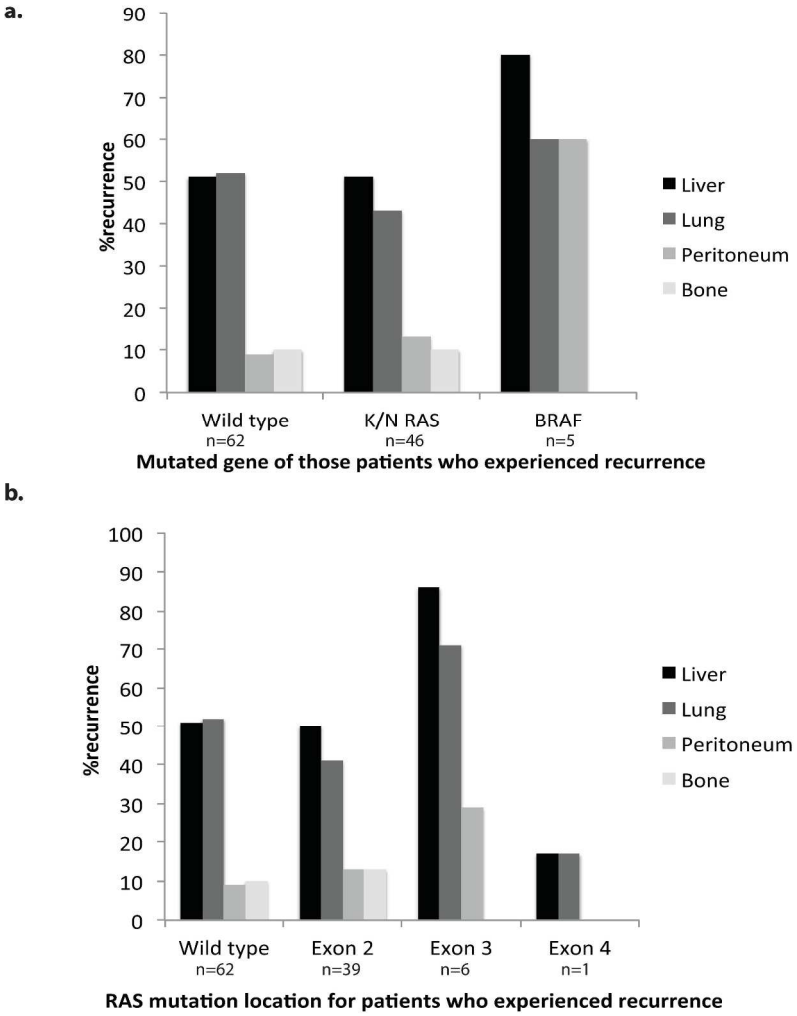


b.



244x292mm (300 x 300 DPI)

Figure 6



264x295mm (300 x 300 DPI)