

Yttrium-90 radioembolization for colorectal cancer liver metastases in KRAS wild-type and mutant patients: Clinical and ccfDNA studies

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Abstract. Patients with unresectable, chemo-refractory colorectal cancer liver metastases (CRCLM) have limited local treatment options. We report our institutional experience on the efficacy of resin-based yttrium-90 (⁹⁰Y) radioembolization for the treatment of CRCLM and our findings on associated circulating cell-free DNA (ccfDNA) studies. A total of 58 patients treated with ⁹⁰Y for CRCLM at the Medstar Georgetown University Hospital had a median survival of 6 months [95% confidence interval (CI), 4.55-7.45 months] after treatment, with a 12-month survival rate of 33%. The median survival from treatment stratified by mutational status was longer in the wild-type (WT) as compared to the KRAS mutant patients at 7 vs. 5 months, but did not achieve statistical significance (p=0.059). Median tumor local control duration after ⁹⁰Y treatment was 2 months (95% CI, 0.34-3.66 months) for the entire cohort and was longer in the WT vs. the mutant patients (2 vs. 1 month, respectively, p=0.088). Plasma was prospectively collected from a subset of 9 patients both before and after single lobe treatment, and ccfDNA concentration and fragmentation index (FI) were measured using quantitative PCR and atomic-force microscopy (AFM). In the WT and KRAS mutant patients, DNA FI was reduced from a median of 0.73-0.65 after treatment. A reduction in DNA FI after single lobe treatment was associated with an improved overall survival (p=0.046). Analysis by AFM of paired pre- and post-treatment samples from KRAS mutant and WT patients

revealed a larger average decrease in fragment size in the WT patients (p=0.013). ⁹⁰Y radioembolization extends local control for CRCLM, however, KRAS mutant tumors may be more radio-resistant to treatment. Changes in the FI of patients following treatment were noted and may be evaluated in a larger study for relevance as a biomarker of response.

Introduction

Colorectal cancer (CRC) is the third most frequent type of cancer diagnosed and the second leading cause of cancer-related deaths in the United States, with an estimated 132,700 new diagnoses and 49,700 deaths in 2015 (1). Approximately 25% of patients present with metastatic disease, while another 25-35% of patients develop metastatic disease during or after treatment, with the liver being the most common site of metastases (2). Surgical resection has been shown to be the only potentially curative option for colon cancer metastasis to the liver (3), resulting in a 25-40% overall survival at 5 years compared to 0-5% in those not having a liver resection. However, only about 15% of patients are considered to be resectable at presentation (2). While improvements in chemotherapy regimens and increasingly aggressive surgical approaches have resulted in improvements in survival rates for metastatic patients (4), patients with unresectable, chemo-refractory colorectal cancer liver metastases (CRCLM) have limited local treatment options. In addition to being prognostic for overall survival, the presence of hepatic disease is the major contributor to the cause of death in approximately half of the metastatic CRC patients (5), indicating that local control of the liver is an important aspect of disease management and outcomes.

For unresectable liver metastases, local therapies include stereotactic body radiation therapy (SBRT), hepatic arterial infusion of chemotherapy, arterially directed embolic therapy, radiofrequency ablation, and liver directed radiation. Selection of these different modalities often depends on the extent, size, and location of the disease (6). Yttrium-90 (⁹⁰Y)

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radioembolization exploits the physiological difference in the blood flow between tumors and the normal liver to preferentially deliver radiation to the metastases, thereby shrinking the tumors while sparing the healthy liver (7). Liver directed radiation has shown promising results in a variety of institutional studies, demonstrating promising survival outcomes and low toxicities (8-10). A recent randomized phase III trial, SIRFLOX, which compared first line chemotherapy vs. the same chemotherapy with selective internal radiation, demonstrated a significant delay of disease progression in the liver (11).

CRC is a heterogeneous disease composed of multiple disease subtypes (12). While about 25% of CRCs are associated with a family history, suggesting a role for inherited genetic mutations, only a small percentage of family history-linked cancers are associated with highly penetrant mutations in major genes. The majority of CRCs occur sporadically, developing in a multi-step process, involving an accumulation of mutations in tumor-suppressor genes and oncogenes (13). Mutations in KRAS have been associated with 33-48% of CRCs. The KRAS protein is a downstream effector of EGFR receptor tyrosine kinase activity, which activates intracellular signaling cascades mediated by the RAF/MEK, MAPK, AKT and PI3K pathways (14). Mutations in KRAS have been linked to CRC progression, with KRAS mutants having worse progression-free survival and overall survival compared to wild-type (WT) patients (13,15-17). The impact of KRAS mutation on radiation response is less clear, with one group reporting a greater survival benefit after ⁹⁰Y radioembolization in WT vs. KRAS mutant patients. However, as there was no evaluation of local control after therapy, the differential assessment of radiation response in the two groups remains unclear (18).

The presence of abnormally high levels of circulating cell-free DNA (ccfDNA) in the plasma of cancer patients has been well established and is thought to result from a variety of different mechanisms including apoptosis, necrosis, and circulating tumor cell lysis (19,20). In CRC patients, an elevated plasma DNA concentration has been shown to be diagnostic for benign vs. malignant disease and to correlate with worse progression-free survival and overall survival after chemotherapy (21-23). DNA fragmentation index (FI), which is measured as the ratio of longer to shorter DNA fragments, has been shown to be higher in patients with advanced stages of breast cancer and with post-operative recurrence (24). In addition, recently, the level of ccfDNA fragmentation has also been shown to correlate with survival in metastatic colon cancer patients (22). However, changes in ccfDNA concentration and FI after ⁹⁰Y radioembolization have not been assessed.

Here we report on our institutional experience on the efficacy of resin-based ⁹⁰Y radioembolization for the treatment of CRCLM and the changes in ccfDNA levels and FI with liver directed internal radiation, investigating whether gene mutations in KRAS could serve as prognostic factors for ⁹⁰Y treatment of CRCLM.

Materials and methods

Patient selection. This retrospective review and prospective blood evaluation was approved by the Institutional Review

Board (IRB 2008-344) of Georgetown University. From May 2011 to March 2015, a total of 58 patients underwent ⁹⁰Y treatment for CRCLM at our institution. Patients eligible for study inclusion had histologically-confirmed CRC with liver metastases. Patients were included irrespective of prior treatment, including prior chemotherapy, surgery, or radiation therapy. All patients were not considered candidates for surgical resection and many of the patients were considered refractory to chemotherapy. Lesions were considered for treatment in any location within the liver. Patients generally were expected to have a life expectancy of at least 6 months and adequate hepatic function.

Pre-treatment imaging, planning, and treatment. All patients underwent pre-procedural imaging with computed tomography (CT), FDG PET-CT, and/or MRI to determine liver tumor location, size, and volume. Three dimensional volumetric software was utilized to evaluate the total tumor volume, total liver volume, and total liver tumor burden (ratio of TV/LV). A pre-procedure planning angiogram and embolization of extrahepatic arteries at risk of nontarget perfusion of ⁹⁰Y microspheres was performed. Hepatopulmonary shunt estimation was performed using Tc-99m-MAA, and this was judged to be adequate if <20%. Therapy with ⁹⁰Y microspheres was performed within 2 weeks of initial planning angiography. ⁹⁰Y microsphere infusion followed the manufacturer's guidelines. Following delivery, a SPECT of the abdomen was performed to assess the distribution of the microspheres. The patients were discharged home the same day.

Depending on extent of disease within the liver at presentation, patients received either bilobar (right and left lobe) or unilobar treatment. For bilobar treatment, the first lobe treatment was followed by the second lobe treatment approximately 30 days later. Infusion of ⁹⁰Y microspheres was performed by both the interventional radiologist and radiation oncologist. Patients were treated to a mean dose of 43.87 Gy and mean activity of 24.28 mCi.

Plasma isolation, ccfDNA extraction, qPCR, and AFM preparation. Sample handling was in accordance with published NCI biospecimen best practices. A 10 ml sample of blood was collected in a K2-EDTA tube. Blood samples were processed within 30-60 min with an initial spin at 800 x g for 10 min at 4°C. The plasma was transferred to another tube and centrifuged at 13,000 x g for 10 min to remove the remaining blood cells. The supernatant plasma samples were then aliquoted into 2 ml/tube and either immediately processed for DNA extraction or stored at -80°C. ccfDNA was extracted from 5 ml of plasma using the QIAamp DNA Mini blood kit (Qiagen, Valencia, CA, USA) according to the 'Blood and Body Fluid Protocol'. ccfDNA samples were kept at -20°C until use.

Plasma was prospectively collected from 9 patients both pre- and post-treatment, and ccfDNA concentrations and FI values were determined using quantitative PCR as previously described (25). Briefly, qPCR amplifications were performed in duplicate in a 20- μ l PCR mix, including the amplification primer and DNA extract. Thermal cycling consisted of three repeated steps: a 3-min Hot Start polymerase activation-denaturation step at 95°C, followed by 40 repeated cycles at 95°C for 10 sec and 60°C for 30 sec. Melting curves were obtained

by increasing the temperature from 55 to 90°C with a plate reading every 0.2°C. Serial dilutions of genomic DNA were used as a standard for quantification. The quantity of ccfDNA fragments of different sizes was assessed using an integrated PCR system that targeted intronic sequences within the same regions. The degree of ccfDNA FI was assessed by calculating the ratio of the concentration determined by using the primer set amplifying a large target (>250 bp) to the concentration determined by using the primer set amplifying a short target (<100 bp). These values were normalized, and the ratio of large fragments (~ 250 bp) to small fragments (<100 bp) was recorded as the DNA FI. The ratio of DNA FI values before and after treatment (pre-treatment FI/post-treatment FI) were stratified by values less than or greater than 1.

Samples for atomic-force microscopy (AFM) analysis were prepared as previously described (26). Briefly, a 2 µl ccfDNA sample in a Tris and EDTA solution was deposited onto APS-mica. The sample was then rinsed with deionized water and dried with nitrogen gas. It was mounted on a Nanoscope IIIa AFM for imaging (Veeco/Digital Instruments, Santa Barbara, CA, USA). Images were acquired in tapping mode in air using silicon tapping mode probes. Ten images were acquired for each ccfDNA sample. Acquired AFM images were analyzed using commercially available software, FemtoScan Online (Advanced Technologies Center, Moscow, Russia), for measurements of ccfDNA fragment lengths. Measured DNA fragments were sorted by size, and the fragment size distribution was obtained by dividing the number of fragments in each bin by the total number of fragments. DNA fragment size conversions were made on the assumption that 1 bp was equivalent to a length of 0.34 nm. AFM was performed on 6 patients, 4 KRAS WT and 2 KRAS mutants.

Follow-up and statistical analysis. A retrospective review was conducted for clinical outcomes, demographic information, and tumor mutational status. In 47 patients, interval imaging, including CT, PET-CT, and/or MRI, was available for tumor response assessment using RECIST criteria as a score of complete response, partial response, stable disease, or progressive disease (27). Because use of ⁹⁰Y microspheres is a liver-directed therapy, tumor response was limited to an assessment of hepatic lesions in the liver lobe or lobes treated, with whole liver local control assessed for bilobar treatments and treated liver lobe local control assessed for unilobar treatments. Tumor mutational status was available for 49 patients based on the review of documented pathology reports. Actuarial survival and progression-free survival were evaluated by the Kaplan-Meier method. Univariate analysis was performed using Kaplan-Meier, and multivariate analysis was assessed using Cox regression analysis. A two-tailed t-test was used to assess changes in fragment size with AFM in WT and KRAS mutant patients before and after single lobe ⁹⁰Y treatment.

Results

Patient characteristics. Patient characteristics are noted in Table I. Our patient population consisted of 58 patients diagnosed with colon cancer at a median age of 56 years (range, 31-85 years). Half of the patients were male, 86% had a performance status ≤1, and 67% of the patients had metastatic

Table I. Patient characteristics, n=58.

Patient characteristics	n (%)
Age at ⁹⁰ Y treatment (years)	
Median (range)	56 (31-85)
≤40	3 (5)
>40 to ≤50	13 (22)
>50 to ≤60	16 (28)
>60 to ≤70	16 (28)
>70	10 (17)
Race	
White	36 (62)
Black	12 (21)
Other	10 (17)
Gender	
Male	29 (50)
Female	29 (50)
Performance status (ECOG)	
0	14 (24)
1	36 (62)
≥2	7 (12)
Unknown	1 (2)
Location of lesion treated	
Right lobe	50 (86)
Left lobe	40 (69)
Both lobes	32 (55)
Presence of extrahepatic disease	
Absent	19 (33)
Present	39 (67)

⁹⁰Y, yttrium-90.

disease outside the liver. Patients received ⁹⁰Y treatment for a median of 20 months (range, 5-52 months) after diagnosis with hepatic metastases. As summarized in Table II, our patients received a median of two chemotherapy treatment lines prior to ⁹⁰Y treatment, ranging from 1 to 5. Every patient had exposure to either 5-FU or capecitabine prior to treatment.

As summarized in Table III, tumor mutation information was available for 49 patients, with 27 (55%) being KRAS mutant, 21 (43%) being WT, and 1 (2%) being Her2/neu over-expressing. A larger proportion of KRAS WT patients were treated with anti-EGFR-targeted agents before ⁹⁰Y radioembolization, but the overall exposure to EGFR inhibitors was low (10%, Table II).

⁹⁰Y radioembolization procedure. A total of 90 treatments were delivered to 58 patients (Table I). Eight patients received treatment to the left lobe alone, 18 received treatment to the right lobe alone, and 32 received treatment to both liver lobes spaced approximately a month apart. The delivered activity during ⁹⁰Y treatment ranged from 8.9 to 59.4 mCi.

Imaging response and liver progression-free survival. In 47 patients, interval imaging, including CT, PET/CT

Table II. Chemotherapy characteristics, n=58.

Chemotherapy characteristics	n (%)
No. of previous chemotherapy lines	
1	12 (21)
2	29 (50)
≥3	17 (29)
Exposure to 5-fluoruracil or capecitabine	
No	0 (0)
Yes	58 (100)
Exposure to leucovorin	
No	9 (16)
Yes	49 (84)
Exposure to oxaliplatin	
No	5 (9)
Yes	53 (91)
Exposure to irinotecan	
No	27 (47)
Yes	31 (53)
Exposure to bevacizumab	
No	14 (24)
Yes	44 (76)
Exposure to cetuximab or panitumumab	
No	52 (90)
Yes	6 (10)
Exposure to nilotinib or sunitinib	
No	54 (93)
Yes	4 (7)
Exposure to other agents ^a	
No	44 (76)
Yes	14 (24)

^aIncludes PARP inhibitors, anti-PDL1, docetaxel, C-met inhibitors, and tezacitabine.

and/or MRI, was available for tumor response assessment. Fig. 1 shows representative CT images before (Fig. 1A and C) and after treatment (Fig. 1B and D) in a WT (Fig. 1A and B) and KRAS mutant (Fig. 1C and D) patient. The median time of imaging after the procedure was 2 months following treatment. RECIST criteria were utilized to measure the response in each liver lobe treated (28). Imaging assessment showed a partial response in 9 patients (19%), stable disease in 20 (43%), and progressive disease in 18 (38%).

Median liver lobe progression-free survival duration after ⁹⁰Y treatment was 2 months [95% confidence interval (CI), 0.34-3.66 months] for the entire cohort (Fig. 2A). The median local control duration assessed according to tumor mutation was longer in the WT vs. KRAS mutant patients of 2 vs. 1 month, respectively (p=0.088) (Fig. 2B). While this was not significant with univariate analysis, it was significant with multivariate analysis (p=0.031) (Table IV). Progression-free survival in the liver was not significantly different by univariate or multivariate analysis in patients stratified by prior

Table III. Mutation status, n=49.

Mutation status	n (%)	Median age (years)
KRAS WT	21 (43)	53
KRAS mutated	27 (55)	60
Her2/neu	1 (2)	38

WT, wild-type.

treatment number of chemotherapy lines (Table IV). However, patients with prior exposure to nilotinib or sunitinib had worse progression-free survival in the liver with univariate analysis (5.6±1.3 vs. 0.5±0.5 months, p=0.034), but this was not significant with multivariate analysis (Table V).

Survival outcomes. Of the 58 patients, 46 died during follow-up. As shown in Fig. 3A, the median survival from diagnosis was 35 months (95% CI, 26.9-43.1 months). The median survival after ⁹⁰Y was 6 months (95% CI, 4.55-7.45 months) in the entire cohort (Fig. 3B), with a 12-month survival of 33%. Patients with ECOG performance status ≤1 had significantly improved median survival over those with ECOG ≥2 (7 vs. 4 months, p=0.001) (Fig. 3C and Table IV). In addition, with univariate analysis, patients younger than 60 also had improved survival after treatment (p=0.044) (Table IV). The median survival from treatment stratified by mutational status (Fig. 3D and Table IV) was longer in the WT as compared to the KRAS mutant patients at 7 months (95% CI, 1.8-12.2) vs. 5 months (95% CI, 3.0-7.0), but did not achieve statistical significance (p=0.059). While overall survival from treatment stratified by the number of prior chemotherapy lines was not significant with either univariate or multivariate analysis (Table IV), prior exposure to oxaliplatin was associated with improved survival from treatment (22.5±1.1 vs. 9.0±1.0 months, p=0.009) and prior exposure to nilotinib/sunitinib was associated with a worse survival from treatment (10.5±1.1 vs. 3.8±0.48 months, p=0.01) (Table V). Furthermore, with multivariate analysis, survival after treatment was significantly associated with performance status [p=0.003; HR, 0.24 (CI, 0.09-0.61); Table IV] and prior exposure to oxaliplatin and nilotinib/sunitinib (p=0.039 and p=0.045, respectively; Table V).

Survival from diagnosis was improved in those patients with ECOG performance status ≤1 (42.3±4.5 vs. 27.3±6.5 months, p=0.17; Table IV). With multivariate analysis, both the performance status and the number of previous chemotherapy lines were significant for survival from cancer diagnosis (Table IV). Prior exposure to oxaliplatin was statistically significant for overall survival from diagnosis (70.5±8.8 vs. 37.4±3.8 months, p=0.039) with univariate analysis, but did not maintain significance with multivariate analysis (Table V).

ccfDNA analysis. ccfDNA, which was collected immediately prior to ⁹⁰Y administration and 2-4 weeks after single lobe treatment, was detected in all of the 9 analyzed samples by PCR. In the WT and KRAS mutant patients, DNA FI was decreased from a median of 0.73-0.65 after treatment. As shown in Fig. 4, a decrease in the ratio of DNA FI (pre-treatment FI/post-treatment

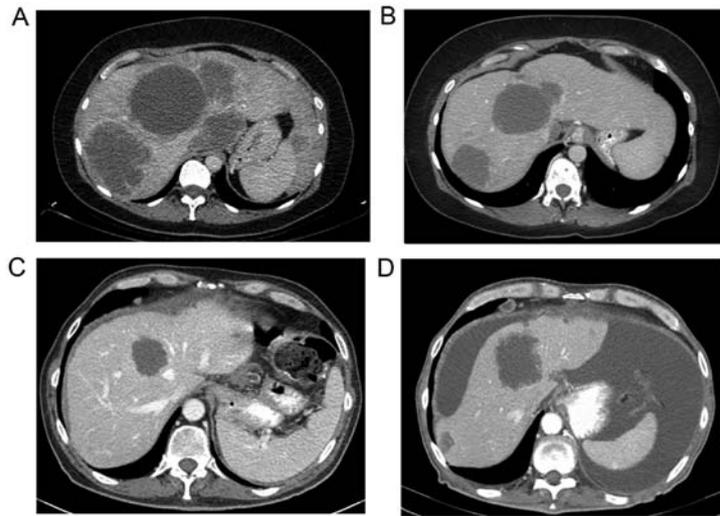


Figure 1. Representative CT images of DNA in (A and B) wild-type and (C and D) KRAS mutant patients before (A and C) and after (B and D) ⁹⁰Y treatment.

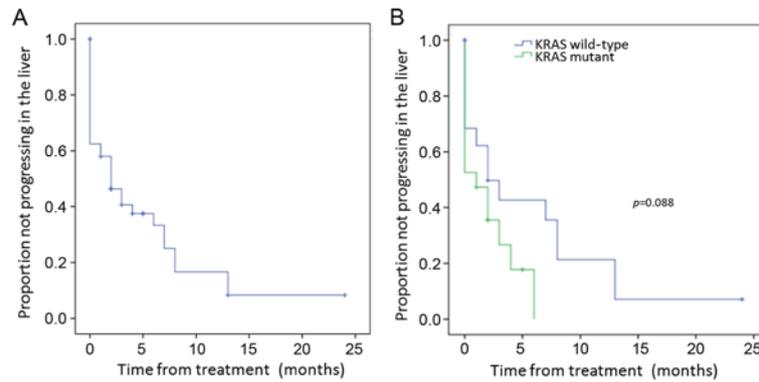


Figure 2. Progression-free survival in the liver: (A) all patients; (B) patients by mutational status.

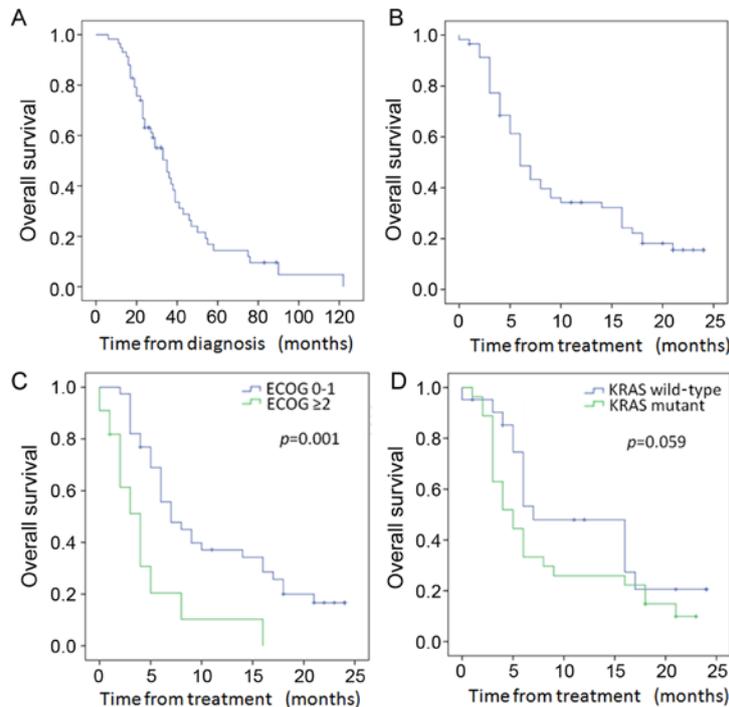


Figure 3. Overall survival: (A) all patients from diagnosis; (B) overall survival from treatment all patients; (C) overall survival of patients by performance status from treatment; (D) overall survival of patients by mutation status from treatment.

Table IV. Univariate and multivariate analyses for progression-free survival and overall survival.

Variables	Reference	Progression-free survival in liver				Overall survival treatment				Overall survival diagnosis			
		Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
		P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)
Age (years)	<60	0.230	2.12 (0.41-10.99)	0.370	0.044	0.82 (0.22-3.09)	0.766	0.598	1.92 (0.47-7.95)	0.366			
Gender	Female	0.065	1.55 (0.60-3.98)	0.364	0.442	0.87 (0.38-1.98)	0.739	0.458	0.82 (0.34-1.94)	0.644			
Performance status	≤1 vs. ≥2	0.395	0.63 (0.13-3.08)	0.568	0.001	0.24 (0.09-0.61)	0.003	0.017	0.21 (0.07-0.62)	0.005			
Smoking	No	0.742	1.24 (0.36-4.27)	0.731	0.444	1.38 (0.51-3.76)	0.524	0.013	2.03 (0.62-6.68)	0.245			
Presence of extrahepatic disease	No	0.445	0.83 (0.30-2.30)	0.723	0.775	0.90 (0.40-2.01)	0.801	0.722	1.36 (0.58-3.18)	0.475			
Mutation status	KRAS WT	0.088	0.29 (0.09-0.90)	0.031	0.059	0.58 (0.26-1.33)	0.200	0.322	0.69 (0.28-1.74)	0.435			
FI	<1	0.716	NA	0.170	0.040	NA	NA	0.040	NA	NA			
No. of previous chemotherapy lines	Continuous		3.87 (0.47-32.16)	0.211		1.11 (0.17-7.18)	0.911		0.2 (0.05-.89)	0.035			
	1 vs. ≥2	0.387	2.96 (0.23-38.73)	0.409	0.652	1.60 (0.19-13.59)	0.667	0.278	0.21 (0.03-1.47)	0.115			
	≤2 vs. ≥3	0.703	5.07 (0.35-73.60)	0.235	0.845	1.00 (0.11-9.28)	0.998	0.801	0.24 (0.04-1.50)	0.128			

FI, fragmentation index; WT, wild-type; NA, not applicable.

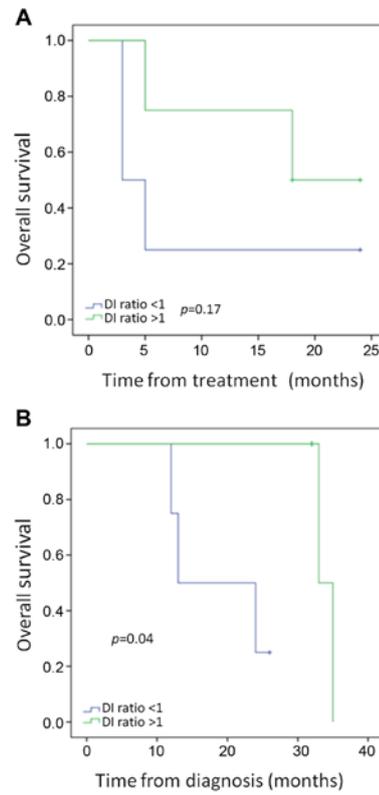


Figure 4. Evaluation of KRAS mutant and wild-type patients for changes in circulating cell-free DNA. (A) Overall survival from treatment according to DNA fragmentation index (FI) changes; (B) overall survival from diagnosis according to DNA FI changes.

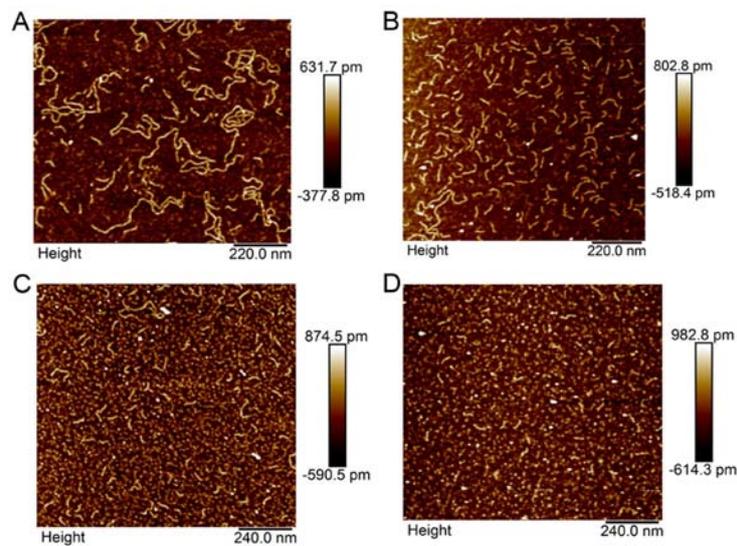
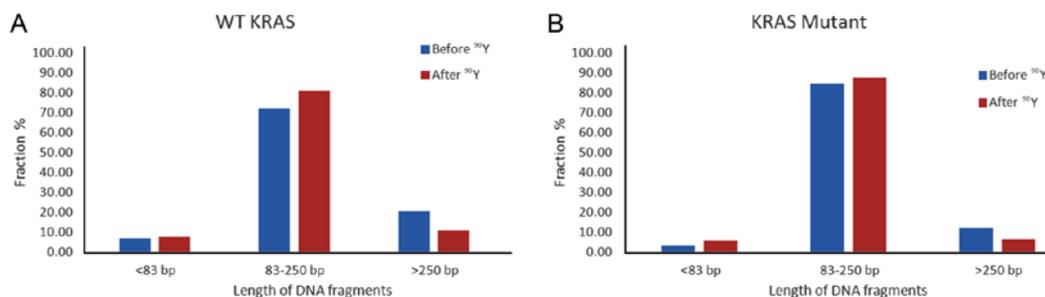
FI), or an increase in the fraction of small DNA fragments, after single lobe treatment was associated with improved outcomes. While overall survival from treatment did not meet statistical significance in patients with a DNA FI ratio pre/post-treatment of >1 vs. <1 (Fig. 4A, $p=0.17$), overall survival from diagnosis was significantly improved in those patients (Fig. 4B, $p=0.04$).

AFM analysis. Analysis by AFM of paired pre- and post-treatment samples was performed in 4 WT and 2 KRAS mutant patients. An illustrative example is shown in Fig. 5 of sample AFM images of WT (Fig. 5A and B) and KRAS mutant (Fig. 5C and D) DNA before (Fig. 5A and C) and after (Fig. 5B and D) single liver lobe exposure to ⁹⁰Y. This figure demonstrates the capability of AFM in measuring individual DNA fragments and illustrates the treatment related differences in WT and KRAS mutant patient ccfDNA.

In a pooled analysis of 4 WT and 2 KRAS mutant patients, the average size of ccfDNA bp change after single lobe treatment was significantly larger in the WT patients, with the WT ccfDNA decreasing from 251.86 to 154.65 bp as compared to the KRAS mutant ccfDNA decreasing from 177.98 to 155.09 bp ($p=0.013$). Quantification of changes in different fragment size groups before and after treatment is shown in Fig. 6. While both WT and KRAS mutant patient ccfDNA had decreased percentage of >250 bp size range and higher proportion of 85-350 bp ccfDNA after single lobe treatment, the magnitude of change was larger in the WT patients, with the 85-350 bp size fragments increasing by 12.3% in the WT (Fig. 6A) and 3.6% in the KRAS mutant (Fig. 6B).

Table V. Univariate and multivariate analyses for progression-free survival and overall survival with chemotherapy exposure.

Variables	Progression-free survival in liver			Overall survival treatment			Overall survival diagnosis		
	Univariate	Multivariate		Univariate	Multivariate		Univariate	Multivariate	
	P-value	HR (95% CI)	P-value	P-value	HR (95% CI)	P-value	P-value	HR (95% CI)	P-value
Oxaliplatin	0.5	0.61 (0.19-2.0)	0.413	0.009	0.12 (0.02-0.90)	0.039	0.039	0.174 (0.02-1.33)	0.092
Leucovorin	0.464	1.35 (0.51-3.55)	0.545	0.063	1.89 (0.84-4.28)	0.127	0.391	0.94 (0.41-2.15)	0.886
Irinotecan	0.514	0.79 (0.34-1.81)	0.569	0.689	0.71 (0.38-1.34)	0.291	0.114	1.28 (0.64-2.56)	0.479
Bevacizumab	0.073	0.39 (0.12-1.32)	0.132	0.426	1.36 (0.68-2.75)	0.385	0.858	1.0 (0.50-2.0)	0.988
Cetuximab or panitumab	0.799	0.56 (0.17-1.84)	0.338	0.708	1.34 (0.46-3.87)	0.59	0.332	1.65 (0.55-4.93)	0.372
Nilotinib or sunitinib	0.034	0.45 (0.15-1.34)	0.15	0.01	0.32 (0.11-0.97)	0.045	0.1	0.47 (0.16-1.41)	0.176

Figure 5. Representative atomic-force microscopy images of DNA in (A and B) wild-type (WT) and (C and D) KRAS mutant patients before (A and C) and after (B and D) yttrium-90 (⁹⁰Y) treatment. ⁹⁰Y treatment appeared to induce significant fragmentation in circulating cell-free DNA for KRAS WT but not in KRAS mutant patients.Figure 6. Comparison of the fractional fragment size distribution of circulating cell-free DNA (ccfDNA) from (A) wild-type (WT) and (B) KRAS mutant samples before and after single lobe treatment with yttrium-90 (⁹⁰Y). The estimated amount of each ccfDNA fraction expressed as the percentage of the total ccfDNA estimated as the sum of the ccfDNA amount of the three size fractions in (A) WT and (B) KRAS mutant patients before (blue) and after (red) treatment in (A) four pooled WT samples and (B) two pooled KRAS mutant samples.

Discussion

In this study we demonstrated that KRAS mutant patients had less radiation-related changes in ccfDNA after treatment than

WT patients and that radiation treatment changes in ccfDNA, as measured by DNA FI, were predictive for patient overall survival. While several studies have previously described the predictive potential of ccfDNA levels in overall survival after

chemotherapy for metastatic CRC patients (23,29), this is the first study to demonstrate the treatment FI ratio as a potential predictive marker for survival.

Molecular biomarker research has revealed CRC to be a heterogeneous disease with multiple molecular disease subtypes (12). CRC biomarker research has led to the development of new chemotherapy regimens and novel monoclonal antibody therapies targeting critical molecules in cell signaling pathways, and these treatment advances have resulted in improvements in overall survival for metastatic CRC (4). There is evidence that tumor response to chemotherapy is predictive for overall survival. In a study by Adam *et al.*, response to chemotherapy correlated with improved overall survival in patients who received neoadjuvant chemotherapy prior to curative liver metastatic tumor resection (30). Mechanisms of resistance to targeted therapies are abundant, with the majority of KRAS WT tumors not responding to anti-EGFR therapy (31). Anti-EGFR therapy resistance has been associated with other gene mutations, epigenetic silencing, and augmented expression of other receptor tyrosine kinases, resulting in a complex tumor microenvironment that may be difficult to predict treatment response (12).

Multiple clinical trials have confirmed the lack of clinical benefit of anti-EGFR therapy against RAS mutant tumors (32,33). The meta-analysis from Sorich *et al.* in 2015 demonstrated that tumors without RAS mutations exhibited an improved clinical response compared to RAS mutant tumors, regardless of the specific targeted therapies or partner chemotherapy used (34). In addition, KRAS mutant tumors have been associated with poor survival outcomes and treatment responses in the absence of targeted agents (16,35,36), which may indicate that these tumors are less responsive to systemic therapies. The impact of KRAS mutation status on radiation treatment response is less clear, but is an area of investigation. Lahti *et al.* reported KRAS mutation status to be predictive for overall survival after ⁹⁰Y radioembolization for unresectable CRC liver metastases (18). While overall survival after ⁹⁰Y treatment in KRAS mutant vs. WT patients did not quite meet statistical significance in our study, there was a clear separation in the curves. Notably, mutation status was significant on multivariate analysis for liver progression-free survival in our study. Intriguingly, it is possible that the same differential chemotherapy responses of WT and KRAS mutant tumors that have been well described are also true for radiation treatment.

El Messaoudi *et al.* recently demonstrated that higher ccfDNA concentrations, higher levels of mutant ccfDNA, and the level of ccfDNA fragmentation correlated with shorter overall survival in metastatic CRC patients (22). While there was no difference in overall survival from treatment as a function of DNA integrity index in our study, analysis of survival from diagnosis was statistically longer in those patients with a decrease in their fragment ccfDNA size after treatment. The discrepancy in survival may be a function of the small number of patients in our study. However, it may also be a function of the limitations of ⁹⁰Y in treating metastatic CRC, which have been previously outlined in the recently published SIRFLOX study (11), with the overall aim of ⁹⁰Y treatment being to control liver disease and not to control systemic burden. Radiation treatment-related DNA FI changes

are likely reflective of tumor response capability. Indeed, DNA FI may reflect tumoricidal changes and could serve as a biomarker to assess treatment response.

Our study sample with molecular data is small. While our findings are hypothesis driving, definitive conclusions are limited until a more robust analysis can be performed on a larger number of patients. In addition, while all of our patients received chemotherapy prior to ⁹⁰Y treatment and many were considered chemotherapy refractory, our patient population was diverse in their overall level of health and in the natural history of their disease. It is not surprising that overall survival was improved in patients with better performance statuses. While no overall survival differences were observed among patients with or without extrahepatic disease, the overall survival differences seen based on the number and types of chemotherapy exposures likely reveals variation in patient performance status and disease course. Further molecular studies on a larger group of patients are necessary to validate our observational findings.

In conclusion, ⁹⁰Y radioembolization is an effective treatment for CRCLM in extending local control for liver dominant metastatic disease. However, KRAS mutant tumors may be more radio-resistant to treatment. In this study, changes in ccfDNA FI were correlated with overall survival, likely indicating that these changes are reflective of treatment response. Measurements of FI may have the potential to be another molecular biomarker that could predict treatment response to therapy.

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