Delayed MRI Enhancement of Colorectal Cancer Liver Metastases Is Associated With Metastatic Mutational Profile

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Abstract. *Background/Aim:* Individual tumor genomics plays a key role in determining patient prognosis, response to chemotherapy and in guiding therapy. In prior studies, it was shown that the degree of late enhancement of colorectal liver metastases (CRCLM) target tumor enhancement (TTE) as seen on magnetic resonance imaging (MRI) was associated with overall survival. In order to better understand the relationship between MRI enhancement and survival, the aim of this study was to characterize genomic profiles of tumors clustered by MRI TTE, and investigate the association between TTE and genetic mutations. *Materials and Methods:* Matched tumor and normal tissue samples from patients with weak TTE and strong TTE were analyzed by Next-generation sequencing (NGS) technology using a custom colorectal cancer panel. *Results:* We discovered a total of 42 non-synonymous somatic mutations from 10 patients with weak TTE and 26 with 10 patients with strong TTE. Adenomatosis Polyposis Coli (APC) was the most commonly altered gene, 18 of those APC mutations were found in the weak TTE and 9 in the strong TTE group. *Conclusion:* An association exists between TTE and mutational status of CRCLM, which may offer some explanation as to why TTE is associated with overall survival in patients with CRCLM.

Colorectal cancer (CRC) is the 2nd leading cause of cancer-related deaths in North America (1, 2). Most deaths are related to metastatic disease, usually to the liver. Despite developments in new treatment techniques, and especially in surgery associated with neoadjuvant or adjuvant chemotherapy, there remains a large range of survival and response to standard treatments between different individual patients, likely due to the “biology” (or subtypes) of individual cancers (3).

There is a growing body of evidence that individual tumor biology that can be distinguished through histology and genetic signatures is playing a key role in determining patient prognosis, response to chemotherapy and in guiding therapy (4-9).

Incorporating these markers into clinical practice is limited by the requirement of direct tissue sampling of the metastases within the liver, which is invasive, costly, and prone to under-sampling (10). Furthermore, tissue characterization is mostly determined via histology of the primary cancer which does not adequately extrapolate to metastatic disease that is by nature heterogeneous and susceptible to mutation over time (11-15).

An ideal prognostic biomarker would provide metastases-specific characterization using non-invasive techniques amenable to repeated measurement and be widely available in Centers that manage colorectal cancer. It would both help choose the patients best suited to undergo surgery and help optimize their pre- and peri-operative treatment and guide chemotherapy in inoperable patients. Magnetic resonance imaging (MRI), is a non-invasive imaging method that is generally accepted as the best imaging method for diagnosis and staging of colorectal liver metastases (CRCLM) (16, 17).

In a prior study, it was shown that the degree of enhancement of the CRCLM, the MRI target tumor enhancement (TTE), were associated with overall survival following liver surgery (18, 19). At 3 years, 88.4% of patients with high TTE on the

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Figure 1. Three examples of patients with colorectal cancer liver metastases (white arrows) demonstrating weak (top panel) and strong (bottom panel) target tumour enhancement (TTE) on 10-minute delayed phase magnetic resonance imaging (MRI) with gadobutrol.

preoperative MRI were alive *vs.* 58.8% in patients with low TTE. TTE was an independent marker of survival in Multivariate Cox-Regression statistics. In this study, the association between TTE and histology was estimated, with a positive association being shown between TTE and fibrosis and a negative association being seen between TTE and necrosis. However, the potential association between TTE and tumor mutation was not investigated. Accordingly, the purpose of this study was to perform next-generation deep sequencing of tumors clustered by TTE, and investigate the association between TTE and mutations.

Materials and Methods

This retrospective study was approved by the Institutional Review Board and need for informed consent was waived.

Specimens. The patients from this study were part of a retrospective cohort, which included all patients at a single tertiary cancer center

with CRCLM who had received a gadobutrol-enhanced MRI after treatment with chemotherapy (variable regimens as determined by standard of care, clinical treatment) and prior to hepatic resection for curative intent between January 1, 2006, and December 31, 2012. The details of the cohort and calculation of TTE were described previously (19).

Tumors were clustered based on their individual TTE. Matched tumor and adjacent normal tissue samples from 10 patients with weak TTE (contrast-to-noise ratio (CNR): -111.2 to -10.8) and 10 patients with strong TTE (CNR: 15.9 to 74.0) were selected from the cohort for the genomic analysis (Figure 1 and Table I). The choice of the tumor was performed based on TTE only and blinded from clinical information, survival and histopathologic analyses.

Genomic DNA extraction. Four 5 μ m sections were cut from the formalin-fixed, paraffin-embedded (FFPE) tissue block of colorectal cancer liver metastases and normal liver from post-hepatectomy pathology specimens. One section was Hematoxylin and Eosin (H&E) stained and area of tumor was marked under microscope. This H&E slide was used as a guide slide for genomic DNA (gDNA) extraction from unstained sections. The gDNA was extracted by the

Table I. List of Study ID, contrast-to-noise ratio (CNR) of the target lesion and Magnetic resonance imaging (MRI) phenotype (weak versus strong target tumor enhancement) used in this study.

Study ID	Lesion (CNR)	MRI Category
249	-111,1951	Weak
15	-104,7803	Weak
659	-46,9107	Weak
292	-40,4455	Weak
568	-47,8899	Weak
643	-35,8564	Weak
391	-67,0529	Weak
778	-28,7722	Weak
288	-10,531	Weak
314	-10,8065	Weak
651	15,9208	Strong
531	19,4601	Strong
344	16,0294	Strong
8	22,4848	Strong
649	46,2663	Strong
545	51,5411	Strong
173	71,1571	Strong
558	55,4951	Strong
409	73,659	Strong
371	74,0232	Strong

MagMAX FFPE DNA/RNA Ultra kit (ThermoFisher Scientific Inc, Waltham, MA, USA). All procedures were carried out in accordance with the manufacturer's instructions. The extracted gDNA was quantified by Qubit 3 Fluorometer with Qubit dsDNA HS Assay Kit. The amount of amplifiable gDNA was determined by the TaqMan RNase P assay (ThermoFisher Scientific Inc).

Targeted exome sequencing. The targeted exome sequencing was performed on the Ion S5XL next-generation sequencing system with a custom designed Colorectal Cancer Gene Panel (ThermoFisher Scientific Inc) (Table II). The custom panel was designed to cover high mutation frequency in Colorectal Cancer from various genomic databases such as Catalogue Of Somatic Mutations in Cancer (COSMIC) database (<https://cancer.sanger.ac.uk/cosmic/>), cBioPortal (<http://www.cbioportal.org/>) and IntOGen (<https://www.intogen.org>). This panel contains 270 primer pairs in 3 pools and covers all coding regions of APC, TP53 and ARID1A genes, and hotspot region of 11 genes (AKT1, ATM, BRAF, CTNNB1, EGFR, FBXW7, KRAS, NRAS, PIK3CA, PTEN and SMAD4) (Table II). The amplicon library was constructed from 30 ng of amplifiable gDNA by Ion Ampliseq Library Plus Kit. The targeted areas were amplified by polymerase chain reaction for 20 cycles. The resulting amplicons were treated with FuPa reagent to partially digest primers. Amplicons were ligated to Ion P1 and Ion Xpress barcode adapters and purified using Agencourt AMPure XP reagent (Beckman Coulter). Barcoded libraries were quantified using the Ion Library TaqMan Quantitation Kit (ThermoFisher Scientific Inc) and diluted to a final concentration of 65pM. The sequencing template preparation was done using Ion Chef with Ion 540 Chef Kits. Sequencing was performed for 500 flows on an Ion S5XL Sequencer with Ion 540 chip.

Table II. Ion Ampliseq Custom Colorectal Cancer Panel.

Gene	Coverage
APC	99.9% of full CDS
ARID1A	96.2% of full CDS
TP53	100% of full CDS
AKT1	Hotspots
ATM	Hotspots
BRAF	Hotspots
CTNNB1	Hotspots
EGFR	Hotspots
FBXW7	Hotspots
KRAS	Hotspots
NRAS	Hotspots
PIK3CA	Hotspots
PTEN	Hotspots
SMAD4	Hotspots

CDS: Coding sequence.

Next generation sequencing data analysis. The Ion Torrent platform-specific pipeline software, Torrent Suite version 5.0.5 (Thermo Fisher Scientific Inc) was used to separate barcoded reads and to filter and remove poor signal reads. The coverage analysis was performed using the coverage analysis plug-in v5.0.4. BAM format files were generated from the sequencing results and then exported to the Ion Reporter Server (ThermoFisher Scientific Inc). The bioinformatics analysis of the sequencing was performed with Ion Torrent platform specific bioinformatics software, Ion Reporter version 5.6. The Ion Reporter Tumor-Normal workflow was used for detection of single nucleotide polymorphisms (SNPs), insertions and deletions in the tumor and normal samples that calculated the intersection of variants in the 2 samples. The workflow performs a statistical evaluation of the likelihood that the tumor allele is not present in the normal sample and calculates a *p*-value representing the statistical confidence of that call. The detected somatic mutations were verified in the Integrative genome viewer (IGV) from the Broad Institute (<http://www.broadinstitute.org/igv/>). The ion reporter workflow also annotates the variants with information from dbSNP, ExAC, 1,000 genomes, 5,000 exomes, OMIM, COSMIC, Polyphen, and ClinVar.

Sanger sequencing validation. Sanger sequencing-based validation was done on mutations in APC and PIK3CA genes detected by the custom designed Ion Ampliseq custom Colorectal Cancer Panel. The nucleotide sequences of the primers in the custom panel were obtained from ampliseq.com. The M13 forward primer sequence 5'TGTA AAACGACGG CCACT-3' was added to the 5' end of the forward primer sequence, and the M13 reverse primer sequence 5' CAGGAAACAGCTATGACC-3' was added to the 5' end of the reverse primer sequence (Integrated DNA Technologies, Coralville, IA, USA). One ng of FFPE DNA was used for amplification of target region. Sanger sequencing was performed at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children, Toronto, Canada (Figure 2). The sequencing data was analyzed by FinchTV version 1.4.0 (Geospiza Inc.).

Microsatellite instability (MSI) analysis. Microsatellite instability status was assessed using two mononucleotide markers (BAT-25 and

Figure 2. Sanger sequencing validation of *PIK3CA* (left panel) and adenomatosis polyposis coli (*APC*) mutations (right panel). Three *PIK3CA* mutations (Met1043Leu, His1047Arg and His1048Arg) in Study ID 391 and *APC* mutation (His913fs) in Study ID 643 were validated by Sanger Sequencing and compared with next generation sequencing (NGS) data. Upper panel shows NGS data viewed in the Integrative genome viewer, middle panel shows Sanger sequencing data viewed in the Finch TV software, lower panel shows Locus, amino acid changes and Nucleotide changes of the mutations.

BAT-26) and three dinucleotide markers (D5S346/APC, D2S123 and D17S250/MFD15) based on their high sensitivity as markers for cancer microsatellite instability status. Primers were fluorescently labelled and PCR amplified fragments were analyzed on an ABI 3500 XL Genetic Analyser using GeneMapper Software 5.0 (Applied Biosystems, Carlsbad, CA, USA) (Table III). The aggregate results of the 5 loci were compared between tumor and normal DNA and interpreted as microsatellite stable (MSS, 0 of 5 loci unstable) or low frequency microsatellite instability (MSI-L, 1 of 5 loci unstable) or high frequency microsatellite instability (MSI-H, 2 or more of 5 loci unstable). A partial (>50%) to complete signal loss of one heterozygote allele was indicated as LOH (loss of heterozygosity) (Table III). All procedures were performed at the molecular pathology laboratory at the Sunnybrook Health Sciences Centre.

Statistical analysis. The number of germline and somatic Adenomatosis Polyposis Coli (*APC*) mutations were compared between TTE groups using Kruskal-Wallis test. The number of patients in each group harboring 2 or more *APC* mutations, as well as other specific mutations detected in our custom panel (TP53 and ARID1A genes and hot spot regions of AKT1, ATM, BRAF, CTNNB1, EGFR, FBXW7, GNAS, KRAS, NRAS, PIK3CA, PTEN and SMAD4) were compared with Mann-Whitney and/or *t*-test depending on the distributions. *p*-Value <0.05 determined statistical significance.

Results

In this study, we carried out genomic profiling of tumors clustered by TTE, and investigated the association between TTE and identified mutations. CRCLM and normal liver from post-hepatectomy pathology specimens were used for next generation sequencing. For variant detection, the mean read depth of 8,693X (between 6,041 and 15,025X) for tumor tissue and 9,280X (between 5,628 and 14,368X) for adjacent normal tissue over the targeted genomic regions were achieved.

A total of 42 non-synonymous somatic mutations were detected from 10 patients with weak TTE (mean of 4.2 mutations) (Table IV) and a total of 26 non-synonymous somatic mutations were detected from 10 patients with strong TTE (mean of 2.6 mutations) (Table V) when compared to respective normal tissue samples. The mutation burden between weak TTE and strong TTE tumors was statistically different ($p=0.0137$). *APC* was the most commonly altered gene, 30 mutations in 17 patients (85%) followed by 11 *KRAS* mutations in 11 patients (55%) and 10 *TP53* mutations in 10 patients (50%). There were no hotspot mutations detected in the *CTNNB1* and *ATM* genes in this study.

Table III. Magnetic resonance imaging (MRI) phenotype (weak versus strong target tumor enhancement) and microsatellite instability (MSI) status of each case.

MRI category	Study ID	MSI status	MSI markers				
			BAT-25	BAT-26	D5S346	D2S123	D17S250
Weak TTE	249	MSI-H			LOH	LOH	LOH
	15						
	659						
	292						
	568	MSI-L			POS		
	643						
	391	MSI-H				POS	POS
	778	MSI-L				POS	
	288						
	314						
Strong TTE	651	MSI-L			POS		
	531						
	344						
	8						
	649	MSI-H					
	545						
	173				POS	POS	
	558	MSI-L					
	409					POS	
	371						

Among a total of 30 mutations detected in APC, 27 mutations (90%) were either nonsense or frameshift mutations by insertion or deletion which are predicted to cause protein truncation or nonsense-mediated decay. Of 27 truncation mutations, 18 mutations (66.6%) are found in the weak TTE and 9 mutations (33.3%) in the strong TTE group. There was also a significant difference in the number of patients with double truncation mutations in APC between the weak TTE group (8/10=80%) and the strong TTE group (2/10=20%) using the Fisher's exact test ($p=0.01$). Two cases in the weak TTE group carried single truncation APC mutations however allele mutation frequency of these mutations were relatively high at 76.6 and 71.3%, respectively. All hotspot mutations in PIK3CA, PTEN, NRAS, BRAF, SMAD4, AKT1 and FBXW7 genes were only found in weak TTE group, however, the presence of KRAS hotspot mutations was not associated with either weak TTE (6/10) or strong TTE group (5/10) (Tables IV and V).

In contrast, 80% of patients (8/10) with strong TTE tumors had TP53 mutations compared to 20% of patients (2/10) with weak TTE tumors. TP53 mutations in strong TTE were also strongly associated with APC mutations (87.5%) (Tables IV and V). Microsatellite instability (MSI) analysis on the 10 weak TTE and the 10 strong TTE cases identified two MSI-H and two MSI-L cases in the weak TTE group compared to one MSI-H and two MSI-L cases

in the strong TTE group. This result indicated that MSI status was not associated with either weak TTE or strong TTE. Interestingly, the two weak TTE cases which possessed a single APC truncated mutation at high mutation frequency (76.6 and 71.3%) also carried microsatellite instability at APC loci (Table III).

Discussion

Our results show an association between TTE, mutation burden and mutation type in CRCLM. In our cohort, lesions with a low TTE had a higher mutation burden compared to lesions with high TTE. Similarly, some mutations were only present in the low-TTE group. In a previous study, it was shown that low TTE patients had a worst survival profile compared to high TTE patients, with a difference of around 30% at 3 years post hepatectomy (19). In this study, late gadolinium enhancement (as measured by TTE) was associated with histopathological evidence of tumour fibrosis. It is a well-known phenomenon that fibrosis in other disease processes demonstrate late enhancement on gadolinium enhanced MRI, including cardiac fibrosis following myocardial infarction or other cardiac diseases (20). Tumour fibrosis in CRCLM is a known histological predictor of long-term prognosis (21). It is hypothesized that this mechanism may explain why TTE is associated with

Table IV. Somatic mutations detected in 10 patients with Weak target tumour enhancement (TTE) by Custom Colorectal Cancer Panel.

Study ID	# Locus	Type frequency (%)	Mutation	Gene	Function	Protein	Coding
15	chr1:115256530	SNV	11,99	NRAS	Missense	p.Gln61Lys	c.181C>A
314	chr1:27023371	INDEL	48,7	ARID1A	Non-frameshift deletion	p.Ala160_Ala162del	c.478_486delGCCGTCGCC
778	chr1:27101138	SNV	33,3	ARID1A	Nonsense	p.Gln1474Ter	c.4420C>T
288	chr3:178936082	SNV	40,2	PIK3CA	Missense	p.Glu542Lys	c.1624G>A
391	chr3:178952072	SNV	68,9	PIK3CA	Missense	p.Met1043Leu	c.3127A>C
391	chr3:178952085	SNV	65,2	PIK3CA	Missense	p.His1047Arg	c.3140A>G
314	chr3:178952085	SNV	70	PIK3CA	Missense	p.His1047Arg	c.3140A>G
391	chr3:178952088	SNV	66,8	PIK3CA	Missense	p.His1048Arg	c.3143A>G
659	chr4:153247289	SNV	43,94	FBXW7	Missense	p.Arg505Cys	c.1513C>T
15	chr5:112116592	SNV	44,91	APC	Nonsense	p.Arg213Ter	c.637C>T
778	chr5:112136976	INDEL	35,1	APC	Frameshift deletion	p.Arg244fs	c.731_731delG
288	chr5:112151204	SNV	16,9	APC	Nonsense	p.Arg283Ter	c.847C>T
314	chr5:112164586	SNV	15,7	APC	Nonsense	p.Arg554Ter	c.1660C>T
292	chr5:112170858	SNV	6,11	APC	Missense	p.His652Tyr	c.1954C>T
778	chr5:112173917	SNV	31,3	APC	Nonsense	p.Arg876Ter	c.2626C>T
643	chr5:112174028	INDEL	45,1	APC	Frameshift deletion	p.His913fs	c.2738_2741delATTG
391	chr5:112174096	SNV	19,9	APC	Nonsense	p.Tyr935Ter	c.2805C>A
659	chr5:112174357	INDEL	40,44	APC	Frameshift insertion	p.Thr1023fs	c.3066_3067insA
292	chr5:112174582	INDEL	24,74	APC	Frameshift deletion	p.Val1099fs	c.3292_3293delTG
659	chr5:112175211	INDEL	49,34	APC	Frameshift deletion	p.Glu1309fs	c.3921_3925delAAAAG
292	chr5:112175224	INDEL	24,86	APC	Frameshift deletion	p.Gly1312fs	c.3934_3934delG
15	chr5:112175348	SNV	46,01	APC	Nonsense	p.Glu1353Ter	c.4057G>T
568	chr5:112175390	SNV	71,29	APC	Nonsense	p.Gln1367Ter	c.4099C>T
288	chr5:112175575	INDEL	28,5	APC	Frameshift deletion	p.Gln1429fs	c.4285_4285delC
314	chr5:112175591	INDEL	38	APC	Frameshift deletion	p.Ser1434fs	c.4301_4301delG
249	chr5:112175672	INDEL	76,59	APC	Frameshift deletion	p.Ser1465fs	c.4385_4386delAG
643	chr5:112175672	INDEL	41,4	APC	Frameshift deletion	p.Ser1465fs	c.4385_4386delAG
391	chr5:112175868	INDEL	43,4	APC	Frameshift deletion	p.Pro1527fs	c.4578_4578delT
249	chr7:140453136	SNV	35,94	BRAF	Missense	p.Val600Glu	c.1799T>A
15	chr10:89624284	SNV	46,15	PTEN	Missense	p.Gly20Arg	c.58G>A
778	chr10:8962899	SNV	43,3	PTEN	Missense	p.Lys128Thr	c.383A>C
391	chr12:25378562	SNV	57,3	KRAS	Missense	p.Ala146Thr	c.436G>A
288	chr12:25398284	SNV	30	KRAS	Missense	p.Gly12Val	c.35G>T
314	chr12:25398284	SNV	48,1	KRAS	Missense	p.Gly12Val	c.35G>T
778	chr12:25398284	SNV	59,8	KRAS	Missense	p.Gly12Val	c.35G>T
643	chr12:25398285	SNV	60,7	KRAS	Missense	p.Gly12Arg	c.34G>C
659	chr12:25398285	SNV	59,73	KRAS	Missense	p.Gly12Ser	c.34G>A
249	chr14:105246527	SNV	3,94	AKT1	Missense	p.Arg25Cys	c.73C>T
249	chr14:105246551	SNV	3,59	AKT1	Missense	p.Glu17Lys	c.49G>A
314	chr17:7577123	SNV	45	TP53	Missense	p.Val272Glu	c.815T>A
568	chr17:7578476	SNV	60,67	TP53	Missense	p.Pro152Thr	c.454C>A
15	chr18:48575163	SNV	85,65	SMAD4	Missense	p.Phe119Leu	c.357T>A

survival. The association between TTE and survival was also shown to exist in both surgical and non-surgical CRCLM patients (22). In our cohort, most patients with weak TTE had two or more mutations in APC, whereas strong TTE lesions had most of the time no or single APC mutations.

Recently, APC mutations, initially thought to be ubiquitous amongst colorectal cancers, have received renewed interest as researchers realized that their numbers

and type, as well as their associations with other mutations, were associated with outcomes in patients with colorectal cancer. For example, a recent study demonstrated that harboring two or more APC mutations was significantly associated with lower overall survival compared to none or one mutation (23) and another study showed that the double mutation of APC and PIK3CA negatively affected survival after hepatectomy (24).

Table V. Somatic mutations detected in 10 patients with Strong target tumour enhancement (TTE) by the Custom Colorectal Cancer Panel.

Study ID	# Locus	Type frequency (%)	Mutation	Gene	Function	Protein	Coding
545	chr1:27023402	SNV	5,17	ARID1A	Missense	p.His170Tyr	c.508C>T
344	chr1:27099390	INDEL	22,15	ARID1A	Frameshift deletion	p.Gly1210fs	c.3628_3641delGGG TATCAGCCCAG
371	chr5:112116592	SNV	5,01	APC	Nonsense	p.Arg213Ter	c.637C>T
545	chr5:112170852	SNV	14,81	APC	Missense	p.Glu650Lys	c.1948G>A
558	chr5:112173917	SNV	7,6	APC	Nonsense	p.Arg876Ter	c.2626C>T
173	chr5:112174631	SNV	55,68	APC	Nonsense	p.Arg1114Ter	c.3340C>T
545	chr5:112174909	INDEL	47,14	APC	Frameshift insertion	p.Thr1208fs	c.3618_3619insA
545	chr5:112175211	INDEL	30,91	APC	Frameshift deletion	p.Glu1309fs	c.3921_3925delAAAAG
344	chr5:112175390	SNV	26,28	APC	Nonsense	p.Gln1367Ter	c.4099C>T
173	chr5:112175514	SNV	17,39	APC	Missense	p.Glu1408Val	c.4223A>T
651	chr5:112175523	INDEL	64,1	APC	Frameshift deletion	p.Ser1411fs	c.4233_4233delT
558	chr5:112175639	SNV	18,22	APC	Nonsense	p.Arg1450Ter	c.4348C>T
409	chr5:112175751	INDEL	35,09	APC	Frameshift insertion	p.Leu1488fs	c.4460_4461insTT
651	chr12:25378562	SNV	73,4	KRAS	Missense	p.Ala146Thr	c.436G>A
558	chr12:25380275	SNV	15,36	KRAS	Missense	p.Gln61His	c.183A>C
409	chr12:25398284	SNV	36,25	KRAS	Missense	p.Gly12Val	c.35G>T
344	chr12:25398284	SNV	24,11	KRAS	Missense	p.Gly12Asp	c.35G>A
649	chr12:25398285	SNV	17,4	KRAS	Missense	p.Gly12Ser	c.34G>A
651	chr17:7577094	SNV	49,7	TP53	Missense	p.Arg282Trp	c.844C>T
371	chr17:7577117	SNV	5,96	TP53	Missense	p.Val274Asp	c.821T>A
173	chr17:7577141	SNV	66,67	TP53	Missense	p.Gly266Val	c.797G>T
344	chr17:7577143	INDEL	24,38	TP53	Non-frameshift deletion	p.Leu265del	c.792_794delACT
649	chr17:7578212	SNV	6,1	TP53	Nonsense	p.Arg213Ter	c.637C>T
558	chr17:7578272	MNV	20,61	TP53	Missense	p.Gln192_His193 delinsHisAsn	c.576_577delGCinsTA
409	chr17:7578412	SNV	62,6	TP53	Missense	p.Val173Glu	c.518T>A
545	chr17:7578461	SNV	74,85	TP53	Missense	p.Val157Phe	c.469G>T
8	No mutations						
531	No mutations						

With the exception of KRAS mutations, found in both groups, hot spot mutations, known for their negative association with survival (8), were only found in weak TTE lesions. On the other hand, TP53 mutations, which seem to be associated with a better outcome (8, 9) were found mostly in strong TTE patients. MSI status was not associated with either TTE group in this sample. Based on these early results, it seems that the lesions with weak TTE were harboring mutations linked to poorer outcomes.

There are limitations to our pilot study. Firstly, the sample size was small, and analysis of a much larger sample would be necessary in order to confirm these results. Secondly, lesions were selected based on their TTE, and not randomly chosen in the cohort. Indeed, only lesions with clearly low TTE and clearly high TTE were included. This was decided in this preliminary study to ensure that lesions were falling into one of these two groups and not at the margin. Analysis of the entire cohort across all ranges of TTE would be

interesting to better understand the relationship between TTE and mutational status. Next, no attempt was made to correlate TTE, mutations and survival in this cohort. This was done purposely, because the sample size used was very small and the results would have limited value. Analysis of larger cohorts will be necessary to further investigate the association between mutations, TTE and survival in these patients.

In conclusion, these early results suggest an association between TTE and mutational status of CRCLM, which may offer some clues as to why TTE is associated with overall survival in patients with CRCLM. Analysis in much larger cohorts of patients are needed to further investigate and validate these promising preliminary findings.

Conflicts of Interest

The Authors have no financial or personal conflicts of interest to disclose.

Authors' Contributions

H.C. and L.M. designed the study, carried out magnetic resonance imaging (MRI) protocol and analysis, helped with writing the paper and statistical analysis of the data. E.H. qualitatively assessed tissue morphology including fibrosis, necrosis, and viable tumor cells on each representative slide and marked areas of tumors on the H and E slides. C.L. helped with study design and selection of cohort. Y.A. did the next-generation sequencing experiments, data analysis and wrote parts of the paper. A.S. provided the conceptual and technical guidance for NGS work, analyzed the data and revised it critically for important intellectual content.

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