**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.

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Key Words: Colorectal cancer, liver metastasis, gene mutations, magnetic resonance imaging (MRI).
preoperative MRI were alive vs. 58.8% in patients with low TTE. TTE was an independent marker of survival in Mutivariate 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 μm 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
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 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       |

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 AAACGACCG CCAGT-3’ was added to the 5’ end of the forward primer sequence, and the M13 reverse primer sequence 5’ CAGGAAAACAGCTATGACC-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
BAT-26) and three dinucleotide markers (D5S346/TP53, 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 stable). 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, EGF, 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.
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=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=80%) and the 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 MSH-L cases in the weak TTE group compared to one MSI-H and two MSH-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 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 | 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    |        |         |
|              | 778      | MSI-L      |        |        |        |        |         |
|              | 288      |            |        |        |        |        |         |
|              | 314      |            |        |        |        |        |         |
| Strong TTE   | 651      | MSI-L      |        |        |        | POS    |         |
|              | 531      |            |        |        |        |        |         |
|              | 344      |            |        |        |        |        |         |
|              | 8        |            |        |        |        |        |         |
|              | 649      |            |        |        |        |        |         |
|              | 545      |            |        |        |        |        |         |
|              | 173      | MSI-H      |        |        | POS    | POS    |         |
|              | 558      |            |        |        |        |        |         |
|              | 409      | MSI-L      |        |        |        | POS    |         |
|              | 371      |            |        |        |        |        |         |
Recently, APC mutations, initially thought to be limited to the development of colorectal cancer, have been 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 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).

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).
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 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 is 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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634
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