Targeted Cancer Next Generation Sequencing as a Primary Screening Tool for Microsatellite Instability and Lynch Syndrome in Upper Gastrointestinal Tract Cancers

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Running Title: Microsatellite Instability Detection by Cancer Sequencing

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Abstract

**Background:** No consensus guideline has been established for microsatellite instability testing in upper gastrointestinal tract cancers. This study aims to determine whether targeted cancer next generation sequencing can accurately detect microsatellite instability in upper gastrointestinal tract cancers and screen for patients with Lynch syndrome.

**Methods:** In a cohort of 645 upper gastrointestinal tract cancers, targeted next generation sequencing assessed microsatellite instability by identifying characteristic insertion and deletion mutations. Sequencing classification was compared to mismatch repair protein immunohistochemistry. Cancers with microsatellite instability by sequencing were analyzed using a testing protocol to identify patients with Lynch syndrome.

**Results:** Sequencing identified microsatellite instability in 3.6% (23 of 645) of upper gastrointestinal tract cancers, including 28% (8 of 29) of small intestinal and 9% (9 of 97) of gastric carcinomas. In 20 cancers classified as having microsatellite instability, 19 demonstrated loss of expression of MLH1, PMS2, MSH2, or MSH6, and one cancer was indeterminate by immunohistochemistry. In contrast, 52 control cancers demonstrated retained expression of all mismatch repair proteins. Using targeted sequencing as the initial screening test, 1.1% (7 of 645) of patients were identified to have pathogenic germline variants confirming a diagnosis of Lynch syndrome.

**Conclusions:** Targeted cancer next generation sequencing is an accurate first-line test to detect microsatellite instability in upper gastrointestinal tract cancers.

**Impact:** This study provides a proof of concept for the use of targeted next generation sequencing to detect microsatellite instability and screen for Lynch syndrome.
Introduction

Microsatellite instability defines a characteristic genomic phenotype of insertion and deletion mutations in DNA repeat regions. Microsatellite instability is caused by the genetic or epigenetic inactivation of DNA mismatch repair genes MLH1, MSH2, MSH6, or PMS2 and is a feature of some sporadic cancers and cancers associated with Lynch syndrome.

Microsatellite instability is most commonly observed in colorectal and endometrial cancers. Although the overall incidence of upper gastrointestinal tract cancers is lower than that of colorectal and endometrial cancers, recent studies evaluating large cancer datasets have identified significant rates of microsatellite instability in cancers of the stomach and small intestine. Clinical guidelines recommend universal screening for Lynch syndrome in colorectal cancers, but no consensus guidelines currently exist for microsatellite instability testing in upper gastrointestinal tract cancers. The accurate identification of microsatellite instability in upper gastrointestinal tract cancers may directly benefit patients in two ways. Microsatellite instability serves as a biomarker to predict response to immune checkpoint inhibitor therapy in solid tumors, including upper gastrointestinal tract cancers. Patients who have cancers with microsatellite instability may be at increased risk for Lynch syndrome, and patients and affected family members may benefit from genetic counseling and germline testing.

As next generation sequencing technology becomes clinically available for cancer genotyping to identify actionable driver mutations, we hypothesize that targeted cancer sequencing panels can also detect microsatellite instability and be used as a primary screening tool for Lynch syndrome in upper gastrointestinal tract cancers.

Materials and Methods
Patients were prospectively enrolled in an institutional cohort study for cancer genotyping. All participants provided written informed consent for tumor sequencing. This study was approved by the Institutional Review Board of the Dana Farber Cancer Institute and the Partners Human Research Committee.

Targeted next generation sequencing on tumor tissue was performed as previously described. DNA was isolated from formalin-fixed, paraffin-embedded tissue with at least 20% tumor nuclei. Indexed sequencing libraries were enriched for exons of 275 genes (102 specimens) or 298 genes (543 specimens), encompassing 757,787 base pairs or 831,033 base pairs of targeted genome, respectively, using solution-based hybrid capture (Agilent SureSelect; Agilent Technologies, Santa Clara, CA). Massively parallel sequencing was performed using Illumina HiSeq2500 (Illumina, Inc., San Diego, CA). Data analysis was performed using a custom pipeline, including Indelocator (GATK; Broad Institute, Cambridge, MA) for calling insertion and deletion variants.

Microsatellite instability as detected by next generation sequencing was defined as greater than 3 microsatellite indel events per megabase pair in the targeted genome, with events defined as single nucleotide insertion or deletion variants in homopolymeric DNA repeats of 4 or more nucleotides. Cancers with total microsatellite indel events below this threshold were classified as microsatellite stable. This criterion achieved 96% sensitivity and 99% specificity compared to mismatch repair protein immunohistochemistry in colorectal adenocarcinomas.

Immunohistochemistry for MLH1, PMS2, MSH2, and MSH6 protein expression was performed as a case control study. We tested all samples classified to have microsatellite instability with available pathology material. For each case sample predicted to have microsatellite instability, at least two control samples classified as microsatellite stable, matched by diagnosis, were tested. In addition, immunohistochemistry was performed on all samples with greater than 2 but fewer than 3
microsatellite indel events per megabase if tissue was available. Immunohistochemistry and MLH1 promoter methylation analyses were performed per standard laboratory protocol as previously described. (11)

Subsequent germline sequencing was performed using the same targeted next generation sequencing assay on DNA isolated from non-neoplastic, formalin-fixed, and paraffin-embedded tissue. The assay included coverage of coding regions of mismatch repair genes MLH1, PMS2, MSH2, and MSH6.

Results

Patient Characteristics

645 upper gastrointestinal tract carcinomas were sequenced, including 230 from esophagus or gastroesophageal junction, 199 from pancreas, 97 from stomach, 60 from bile duct, 29 from small intestine, 19 from gallbladder, and 11 from ampulla of Vater. 426 patients (66%) were male, and 219 (34%) were female. The median age at the time of testing was 65.3 years (mean 64.2 years, range 19.0 to 93.6 years).

Detection of Microsatellite Instability in Upper Gastrointestinal Tract Cancers by Targeted Sequencing

Microsatellite instability was identified by next generation sequencing in 23 of 645 (3.6%) upper gastrointestinal tract cancers. Cancers classified to have microsatellite instability had a median of 9.2 microsatellite indel events per megabase (mean 10.9, range 3.4 to 22.4). 555 of 622 (89.2%) cancers classified as microsatellite stable had 0 microsatellite indel events. 55 of 622 (8.8%) cancers classified as microsatellite stable had between 0 and 2 microsatellite indel events, and 12 of 622 (1.9%) had between 2 and 3 microsatellite indel events (Figure 1A). Microsatellite instability was seen in 8 of 29 small
intestinal (28%), 9 of 97 gastric (9%), 1 of 60 biliary tract (2%), 3 of 230 esophageal or gastroesophageal junction (1.3%), 2 of 199 pancreatic (1.0%), 0 of 19 gallbladder, and 0 of 11 ampullary cancers (Figure 1B).

Validation of Results with Mismatch Repair Protein Immunohistochemistry

20 of 23 cases classified to have microsatellite instability had available tumor tissue for immunohistochemistry. 19 of 20 (95%) showed loss of expression of at least one mismatch repair protein including 14 with loss of MLH1 and PMS2, 2 with loss of PMS2, 2 with loss of MSH2 and MSH6, and 1 with loss of MSH6. The final case showed an unusual pattern, with nuclear staining of 5% of tumor nuclei for all four mismatch repair proteins, and was interpreted as indeterminate.

In contrast, all 45 controls classified as microsatellite stable by sequencing showed intact staining for MLH1, PMS2, MSH2, and MSH6 (Figure 1C). In addition, all 7 cases with between 2 and 3 microsatellite indel events per megabase (classified as microsatellite stable but near the threshold) and with available tissue showed intact staining for MLH1, PMS2, MSH2, and MSH6.

Identification of Lynch Syndrome in Patients with Upper Gastrointestinal Tract Cancers

For cases with microsatellite instability and available matched normal tissue, we followed a testing algorithm commonly applied to colorectal cancers to identify patients with Lynch syndrome (Figure 2). For cancers with loss of MLH1 and PMS2 expression, MLH1 promoter methylation analysis was performed. Of 10 neoplasms with MLH1 and PMS2 loss and successful promoter methylation analysis, MLH1 promoter methylation was identified in 7.

Non-neoplastic tissue for germline testing was available for 12 of 16 specimens with microsatellite instability and without MLH1 promoter methylation. Pathogenic Lynch syndrome variants were identified in 7 patients, representing 1.1% of upper gastrointestinal tract cancers in our cohort,
including 4 small intestinal, 1 esophageal, 1 stomach, and 1 pancreatic cancers (Table 1, Supplemental Data). Patients with Lynch syndrome had a median age of 69.6 years (range 49.8 to 74.6 years). Upper gastrointestinal tract cancer was the first clinical manifestation of Lynch syndrome in 6 of 7 patients. Colonic adenocarcinoma was the first Lynch syndrome-associated cancer in the other patient.

**Somatic and Second Hit Mutations in Upper Gastrointestinal Tract Cancers with Microsatellite Instability**

We evaluated the tumor sequencing data to identify somatic second hit mutations in mismatch repair genes in patients with Lynch syndrome. In 3 of 7 patients with Lynch syndrome, a second loss of function mutation was identified in the tumor specimen. These mutations included a splice site variant and two nonsense variants. In one patient with an isolated MLH1 exon 13 germline deletion, the tumor specimen showed exon 13 deletion involving both copies of MLH1, and the second hit in this cancer was most likely due to loss of heterozygosity of the MLH1 gene locus. In the other 3 patients with Lynch syndrome, a second somatic mutation was not identified, and the observed variant allele fractions in tumor specimens did not support loss of heterozygosity. This result might be due to limitations of the study, including possibly limited ability to detect loss of heterozygosity in relatively low tumor purity conditions or the presence of sequence or structural alterations in noncoding regions of the gene, which were not assessed by this assay. In two upper gastrointestinal tract cancers with microsatellite instability, the full testing algorithm was completed, and germline testing did not identify a pathogenic Lynch syndrome variant. Tumor testing showed somatic inactivation of mismatch repair genes by mutation or focal gene deletion (Table 1).

**Discussion**

Next generation sequencing has emerged as an effective diagnostic tool in cancer care and is being rapidly adopted into clinical practice.(12) In addition to the identification of oncogenic driver
mutations, sequencing can identify patterns of passenger mutations associated with microsatellite instability. Multiple algorithms have been developed to identify microsatellite instability from sequencing assays,\(^\text{13–15}\) including from targeted panel sequencing of tumor only specimens.\(^\text{10,16}\) Recently, tumor sequencing has been suggested as a replacement for traditional Lynch syndrome screening methods in colorectal cancer.\(^\text{17}\)

Compared to colorectal cancer, Lynch syndrome screening in the upper gastrointestinal tract faces practical and diagnostic challenges. Many upper gastrointestinal tract cancers are diagnosed from fine needle aspiration cytologic preparations or small biopsies, and patients are frequently treated with neoadjuvant therapy before surgical resection. These diagnostic and treatment patterns limit the availability of diagnostic tumor tissue and make accurate interpretation by immunohistochemical methods more difficult. In this setting, targeted cancer sequencing is an appealing alternative for microsatellite instability evaluation. The next generation sequencing assay used in this study has been validated to be performed on as little as 50 nanograms of tumor-enriched input DNA.

Our findings demonstrate accurate microsatellite instability assessment in upper gastrointestinal tract cancers by sequencing, and our protocol identifies microsatellite instability in 3.6% and pathogenic germline Lynch syndrome variants in 1.1% of upper gastrointestinal tract cancers in this cohort. Although the frequency of microsatellite instability is lower than that of colorectal cancers, the overall rates of microsatellite instability and Lynch syndrome in upper gastrointestinal tract cancers are clinically significant. Microsatellite instability is a biomarker for response of solid tumors to immune checkpoint inhibitor therapy,\(^\text{5,18}\) and the identification of microsatellite instability provides a treatment option for patients who have failed other systemic therapies. In addition, the ability to screen for microsatellite instability has implications for a subset of patients with upper gastrointestinal tract cancers and Lynch syndrome. Our study has identified seven patients with Lynch syndrome in an
unselected cohort. These patients are of similar age compared to patients with sporadic upper gastrointestinal tract cancers. Notably, three of seven pathogenic Lynch syndrome variants are missense variants, and two variants involve PMS2. Genetic alterations involving PMS2 have been associated with a moderately increased risk of colorectal and endometrial cancers, and the significance of PMS2-associated Lynch syndrome in cancer risk at other sites is controversial. (19)

Patients with Lynch syndrome and affected family members may benefit from enhanced surveillance for the prevention for additional primary cancers, including increased frequency of colonoscopy screening, consideration of prophylactic hysterectomy for women older than 40 years, and other emerging screening strategies. (20) These potential benefits are in addition to the detection of driver oncogenic alterations, which may provide druggable targets in upper gastrointestinal tract cancers or determine eligibility in clinical trials. (21, 22)

Our findings support the use of targeted cancer sequencing as a first-line screening test in upper gastrointestinal tract cancers to identify microsatellite instability and patients with Lynch syndrome. In laboratories already performing panel sequencing to identify driver mutations, the adoption of a similar protocol may benefit patients with upper gastrointestinal tract cancers.

**Acknowledgments**

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**References**


Table 1: 23 upper gastrointestinal tract carcinomas classified as mismatch repair deficient by targeted panel sequencing, validation with immunohistochemistry, and identification of pathogenic germline variants to diagnose Lynch syndrome. VAF: variant allele fraction. Log2 ratio: relative copy number, where 0 represents copy number neutral and -1 represents one copy loss in a specimen where 100% of cells are affected.

<table>
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<th>Site</th>
<th>Microsatellite Events per Mb</th>
<th>Immunohistochemistry</th>
<th>MLH1 Promoter</th>
<th>Germline VAF or Log2 Ratio</th>
<th>Cancer VAF or Log2 Ratio</th>
<th>Somatic Variant</th>
<th>Cancer VAF or Log2 Ratio</th>
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<td>loss</td>
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<td>loss</td>
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<td></td>
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<td>MLH1 deletion</td>
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<td>intact unmethylated</td>
<td>MLH1 c.2194_2197dup (p.H733Q/p*14)</td>
<td>45%</td>
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<td>MSH2 c.2581C&gt;T (p.Q861*)</td>
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<td></td>
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<tr>
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<td>loss</td>
<td>intact</td>
<td>intact not available</td>
<td>not available</td>
<td></td>
<td>MLH1 c.1906G&gt;C (p.A636P)</td>
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<td>pancreas</td>
<td>5.7</td>
<td>intact</td>
<td>intact</td>
<td>not indicated</td>
<td>MSH2 c.1906G&gt;C (p.A636P)</td>
<td>48%</td>
<td>54%</td>
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Figure Legends

Figure 1: Detection of microsatellite instability by next generation sequencing. A, Number of microsatellite insertion or deletion events per megabase in upper gastrointestinal tract cancers. Cancers with greater than 3 events per megabase were classified as having microsatellite instability. B, Frequency of microsatellite instability in upper gastrointestinal tract cancers, by site. C, Validation of microsatellite instability classification by sequencing compared to immunohistochemistry.

Figure 2: Study protocol for the evaluation of upper gastrointestinal tract cancers to identify patients with Lynch syndrome. Targeted next generation sequencing was performed on 645 upper gastrointestinal tract cancers. Confirmatory immunohistochemistry was performed on cancers classified as having microsatellite instability, and MLH1 promoter methylation analysis was tested for cancers with loss of MLH1 and PMS2 expression. Germline testing was performed on cases suspicious for Lynch syndrome-associated cancers, where non-neoplastic tissue was available.
upper gastrointestinal tract cancers  
\( n = 645 \)

next-generation sequencing panel

predicted microsatellite instability  
\( n = 23 \)

predicted microsatellite stable  
\( n = 622 \)

confirmatory immunohistochemistry

MLH1/PMS2 loss  
\( n = 14 \)

PMS2 loss  
\( n = 2 \)

MSH2/MSH6 loss  
\( n = 2 \)

MSH6 loss  
\( n = 1 \)

indeterminate or material unavailable  
\( n = 4 \)

\( MLH1 \) promoter analysis

methylated  
\( n = 7 \)

unmethylated  
\( n = 3 \)

failed/unavailable  
\( n = 4 \)

germline testing

pathogenic Lynch syndrome variant  
\( n = 7 \)

negative for Lynch syndrome variant  
\( n = 5 \)

unavailable  
\( n = 4 \)

Figure 2
Targeted Cancer Next Generation Sequencing as a Primary Screening Tool for Microsatellite Instability and Lynch Syndrome in Upper Gastrointestinal Tract Cancers


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