Tumor-Based Genetic Testing and Familial Cancer Risk

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As genetic testing on somatic tumor tissue becomes a more routine part of personalized cancer treatment, a growing opportunity arises to identify hereditary germline variants within those results. These germline results can affect future cancer screening for both patients and their family members. Finding this germline information can be complicated as a result of differences between somatic and germline testing processes, nomenclature, and outcome goals (e.g., treatment impact). The goal of this review is to highlight differences between somatic and germline testing and outline a potential guide to allow for appropriate clinical interpretation of somatic testing results in order to better facilitate genetic counseling referrals and confirmatory germline testing.

Tumor characteristics have long guided cancer treatment and follow-up care. As awareness of hereditary cancer risk has grown, correlations between histopathologic tumor features and prevalence of germline cancer risk variants have also been noted. Triple-negative breast cancers are more likely to carry *BRCA1* variants compared to estrogen-positive tumors (Peshkin et al. 2010; Krammer et al. 2017). Specific renal cancer histology may suggest particular hereditary renal cancer risk genes, such as oncocytic chromophobe tumors associated with variants in the *FLCN* gene (Peng and Chen 2018). Markers of mismatch repair deficiency were found to include microsatellite instability and loss of expression of certain proteins on immunohistochemistry (IHC), leading to recommendations

for universal screening protocols for colon and endometrial tumors to screen for Lynch syndrome (de la Chapelle and Hampel 2010).

Recent technologies allow for genomic sequencing of tumor tissue with an increased understanding of the tumor genome and its impact on personalized cancer treatment. A growing number of therapies can target certain somatic variants, and tumor genetic testing is becoming a more integral part of cancer treatment. As this testing becomes more widely used, clinicians must be familiar with the potential for somatic tumor testing to identify hereditary cancer risks and recognize the need to facilitate confirmatory testing through genetic counseling. Knowledge of hereditary cancer risk can help patients determine future screening and provide family

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members with valuable information (Jain et al. 2016).

THE MISMATCH REPAIR PATHWAY AND LYNCH SYNDROME

Detection of hereditary cancer risk often begins with a family history–based assessment and various criteria assist in determining personal and family history most likely to yield informative genetic testing results. However, such criteria can miss at-risk families. For example, in Lynch syndrome, also called hereditary nonpolyposis colorectal cancer syndrome (HNPCC), family history–based guidelines may miss up to 39% of at-risk families (Syngal et al. 2000). Pathogenic variants in these genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) cause mismatch repair deficiency (MMRD) and lead to distinctive tumor characteristics including microsatellite instability (MSI) and IHC-detected loss of protein expression from the relevant genes (de la Chapelle and Hampel 2010), which provide tumor-based avenues to identify at-risk patients, including those missed by family history.

Microsatellite Instability

Microsatellites are common regions of chromosomal DNA with repeating sequences of DNA nucleotides. These repeat sequences can expand in the setting of MMRD, such as those caused by Lynch syndrome, and present with high levels of MSI. Approximately 20% of uterine cancers will be MSI-high, with <10% of those associated with a diagnosis of Lynch syndrome (Hampel et al. 2006). Approximately 15% of colorectal tumors will show high MSI, with about one-third of those having Lynch syndrome (de la Chapelle and Hampel 2010). Not all MSI-high tumors will have Lynch syndrome as other events, particularly somatic hypermethylation of the *MLH1* promoter region and biallelic somatic inactivation of one of the genes associated with Lynch syndrome, can also lead to MSI-high findings (Salvador et al. 2019).

MSI testing has historically been a polymerase chain reaction (PCR)-based panel focusing on five loci identified by the National Cancer

Institute (Boland et al. 1998). Variations have been implemented in many pathology departments as universal screening protocols have become more prevalent. As next-generation sequencing (NGS) technologies have expanded in both the tumor and germline genetic testing setting, the ability to detect microsatellite repeats using NGS methodology has been confirmed to have comparable sensitivity and specificity, allowing this information to be incorporated into some tumor sequencing test reports (Salipante et al. 2014).

Immunohistochemistry

Protein expression testing by IHC is also a highly sensitive screening tool for Lynch syndrome. Cancers that develop because of inactivation of the MMR genes typically show a complete lack of protein expression for those genes within the tumor (de la Chapelle and Hampel 2010). In Lynch syndrome, the patient inherits a single pathogenic variant on one of two alleles inherited from the parents. As part of the tumorigenesis process, the wild-type allele will characteristically become nonfunctional (through an acquired somatic variant or loss of heterozygosity) leading to a lack of protein expression in tumor tissue, whereas normal tissue will retain detectable protein levels because of one functional wild-type allele. Abnormal IHC results can also be acquired, similar to high microsatellite instability, through acquired biallelic somatic variants and hypermethylation of the *MLH1* promoter region (Salvador et al. 2019).

IHC is often the preferred method of routine Lynch screening as it is convenient, inexpensive, and makes use of supplies and expertise readily available in most pathology departments. Also, unlike MSI, IHC allows for more specificity in identifying the MMR gene likely affected. MMR proteins act as heterodimers, with *MLH1* proteins typically pairing with *PMS2* and *MSH2* working with *MSH6* (Boland et al. 2008). Non-functioning *MLH1* genes (whether through promoter hypermethylation, biallelic somatic variants, or germline *MLH1* variants) will typically lead to loss of both *MLH1* and *PMS2* protein expression within tumor cells. Inactivation

Table 1. Simplified summary of tumor microsatellite instability (MSI) and immunohistochemistry (IHC) assessment for Lynch Syndrome (LS)

IHC				MSI	Possible explanation
MLH1	MSH2	MSH6	PMS2		
+	+	+	+	MSS or low	Sporadic cancer Possibly non-Lynch syndrome cancer syndrome
N/a	N/a	N/a	N/a	High	Germline mutation in any LS gene Sporadic cancer (MPH, DSM, or other etiology)
–	+	+	–	N/a	Germline mutation—typically <i>MLH1</i> , rarely <i>PMS2</i> <i>BRAF</i> mutation (colon tumor only) or MPH DSM in <i>MLH1</i> and/or <i>PMS2</i>
+	–	–	+	N/a	Germline mutation—typically <i>MSH2/EPCAM</i> , rarely <i>MSH6</i> DSM in <i>MSH2</i> and/or <i>MSH6</i>
+	+	+	–	N/a	Germline mutation in <i>PMS2</i> , rarely in <i>MLH1</i> DSM in <i>PMS2</i> or (rarely) <i>MLH1</i>
+	+	–	+	N/a	Germline mutation in <i>MSH6</i> , rarely in <i>MSH2</i> DSM in <i>MSH6</i> or (rarely) <i>MSH2</i>

(MPH) MLH1 promoter hypermethylation, (DSMs) double somatic mutations.

of only the *PMS2* gene will often allow expression of the MLH1 protein and PMS2 protein expression alone absent on IHC. Similarly, *MSH2* inactivation will lead to loss of both MSH2 and MSH6 protein expression, whereas inactivation of *MSH6* will only affect MSH6 protein expression. It is important to note that MSH6 protein expression can be decreased compared to pretreatment levels in tissue that has undergone chemoradiation (Goldstein et al. 2017), leading to a false report of absence of MSH6 protein in a biopsy or resection of the treated tissue. The National Comprehensive Cancer Network has summarized common explanations for loss of IHC and follow-up recommendations based on IHC and MSI results, a modified form of which is available in Table 1 (NCCN 1.2018).

Universal MMR Screening

Routine screening of colorectal and endometrial tumors for microsatellite stability and IHC has been shown to increase detection of Lynch syndrome beyond family history-based guidelines and has been encouraged as part of routine care in the treatment of colorectal and uterine cancer (Hampel et al. 2008; EGAPP 2009). Universal screening, and subsequent identification

of Lynch syndrome, also allows for “cascade testing” for family members unaffected by cancer, increasing cost-effectiveness of such testing and providing health benefits to extended relatives (Hampel 2016). MMRD also has a growing impact on cancer treatment with the recent discovery of beneficial immunotherapies in MMRD tumors of various types (Le et al. 2015), supporting the need for even broader MMRD screening.

As both tumor gene sequencing and identification of MMRD have a growing impact on cancer treatment, it has been posited that up-front tumor sequencing may effectively replace screening programs focusing only on IHC and/or MSI testing. Identifying Lynch syndrome through IHC/MSI screening programs can require multiple steps over an extended period of time (i.e., tumor screening, *BRAF* testing, *MLH1* hypermethylation testing, and finally germline testing). A recent study found that test sensitivity improved with up-front tumor testing and allowed for identification of additional treatment-related information, such as *KRAS* status and tumor mutational burden, compiled within one test (Hampel et al. 2018). Feasibility and cost-effectiveness of this method compared to in-house IHC/MSI testing has yet to be determined.

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TUMOR SEQUENCING ANALYSIS AND IMPLICATIONS FOR GERMLINE ALTERATION DETECTION

Somatic gene sequencing analyses are most frequently performed by NGS or massively parallel sequencing. This technology replaced single-target detection assays (e.g., Sanger sequencing) with simultaneous evaluation of many genes, utilizing millions of short nucleic acids sequencing in parallel (Bentley et al. 2008; Shendure and Ji 2008). However, the application of this technology has been inconsistent. In a 2015 survey by a clinical laboratory–focused working group from the Association for Molecular Pathology, responders reported using NGS testing in panels ranging from 1 to 10 genes to >100. A small number of laboratories reported performing exome (12%) or genome (5%) analysis. Although all participants reported small-nucleotide variants (SNVs), or the change of a single nucleotide, there was considerably more variability in reporting of copy number variants (CNVs). Only 35%–37% of participating laboratories analyzed for CNVs (Li et al. 2017).

METHODS OF ANALYSIS (TUMOR GENE SEQUENCING)

Analysis is typically carried out using amplicon-based methods, either through vendor-created tools with varying levels of customization or through targeted enrichment with hybridization capture (Cheng et al. 2015). Amplicon methods use PCR to select for desired sequences by using primers that target a particular gene or stretch of DNA. This process creates short segments of DNA for analysis, called amplicons. Amplicon-based methods without hybridization capture are typically more affordable and require less clinical laboratory investment. They are convenient, particularly for smaller gene panels, but susceptible to artifacts of random sequence mismatch and uneven sequence coverage across areas of interest (Nikiforova et al. 2013; Singh et al. 2013; Lin et al. 2014; Luthra et al. 2014; Tsongalis et al. 2014). Additional challenges arise as certain variants may not be detected due to poor coverage in an amplicon-based sys-

tem or not reported because of quality control methods.

Some analyses use hybridization capture. For these assays, amplicon-based NGS is performed, and then biotinylated probes are used to specifically bind to areas of interest within the desired sequences. These areas may be known sequences with typically poor coverage, areas with pseudogene homology, or any other stretch of DNA for which additional amplicons for analysis may be desired. Once, the probes are bound and pulled down, one is left with a more specific library of sequences of interest. This methodology can create more even sequence coverage and reduce the amount of mismatch artifact. For these reasons, large panels have better performance when utilizing a hybridization capture tool with which more accurate estimates of CNVs can be made. However, this frequently involves significant infrastructure and bioinformatics investment (Cheng et al. 2015). The additive value of this investment may not be viewed as important in the treatment setting based on the goals of the institution and the analysis.

Deletion and duplication analysis using an NGS platform can be challenging. Probes must be selected appropriately to allow for even coverage and reduction of pseudogene homology, which is sometimes very challenging. The ability to detect a single exon deletion is quite good with higher levels of sequencing in the flanking intronic regions. However, most NGS assays used for somatic sequencing focus on exonic regions only. Depending on the bioinformatic tool used, deletions of up to 25 base pairs are easy to analyze provided the whole surrounding area has been sequenced. However, larger insertions and deletions can lead to errors in mapping because the aligner tool, the bioinformatics tool that matches the reported sequence to the expected sequence, must contend with large extra or missing portions of DNA when comparing the two sequences. This can lead to lack of alignment or misalignment of the sequence and therefore a possible missed variant call (Cheng et al. 2015, 2017). Understanding which methodology was used for testing is an important distinction when considering the

ability of a somatic analysis to identify a germline variant.

GERMLINE IMPLICATIONS FROM SOMATIC TESTING

For the clinical genetic counselor or other health-care provider, a primary concern when considering a somatic gene report is whether there are germline implications. The IMPACT program at Memorial Sloan Kettering, which offers paired somatic and germline analysis to participants, found ~15% of unselected individuals harbor a pathogenic germline variant. Discordance between tumor type and cancer susceptibility gene were identified in 60% of individuals, and about one-fourth had loss of the second allele (Schrader et al. 2016), suggesting that paired analysis may be a tool for opportunistic hereditary cancer screening in cancer patients who might not otherwise be identified through personal and family history assessment.

However, most cancer patients in the United States do not receive paired analysis when undergoing somatic testing. Using a somatic report as a screening tool for germline alterations can be complicated. Utilization is made even more overwhelming when considering that the mutational burden of these tumors can be high; more than 100 sequencing alterations and CNVs can be identified within a single sample. Studies have suggested that 20% of patients with ovarian cancer have a somatic gene variant associated with the Fanconi anemia/DNA repair pathway (Kanchi et al. 2014), and *TP53* variants were identified in >40% of all tumors analyzed in multiple organs (Zehir et al. 2017). Considering that the recent American Society of Clinical Oncology (ASCO) guidelines recommend genetic testing for all somatic *BRCA1* and *BRCA2* carriers, the ability to parse out germline alterations is both important and timely.

SOMATIC ANALYSIS COMPARED TO GERMLINE

When looking at a somatic report for germline implications, it is important to identify a few key items: intentional germline filtering, transcript

utilization, tumor heterogeneity and purity, allelic fraction, possible founder variants, genes atypical to the tumorigenesis process in the organ, and tumor mutational burden. Differences in the variant classification process between germline and somatic variants should also be kept in mind.

Filtration

In some cases, tumor analysis intentionally filters out germline variants. Previously, there have been instances in which laboratories felt that patients were not adequately consented to receive incidental germline findings and therefore excluded these variants from the analysis (Mandelker and Zhang 2018). Other laboratories intentionally filter out suspected or known germline variants so that they can report on pure somatic alterations, thought to be most active in the tumor and therefore the best targets for therapy (Raymond et al. 2016; Mandelker and Zhang 2018). Therefore, it is possible that a known pathogenic germline variant may not appear on the somatic report if not thought to be clinically significant for the treatment of the cancer.

Transcript Use

Joint Commission Guidelines recommend that the canonical transcript (determined by consensus, longest coding sequence [CDS] or longest cDNA as listed in Ensembl [Zerbino et al. 2018] and University of California, Southern California (UCSC) Genome Browser [Kent et al. 2002]) be used for somatic as well as germline analysis (Li et al. 2017). However, because of the large number of aberrations in the somatic gene setting, there may be discrepancies between nucleotide and amino acid nomenclature when compared to HGVS nomenclature for that transcript at that location (Dagleish et al. 2010). For example, a recent somatic report found a *MUTYH* pathogenic variant called c.1138delC (p.A382fs) at an allele frequency of 57%. The variant was confirmed to be present in the germline, but the nomenclature was different, c.1147del (p.Ala385Profs*23). Many publicly

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available databases, such as ClinVar (Landrum et al. 2018), will list aliases, and alternate locations may be included as a known alias. However, if discrepancy continues to exist, it may be beneficial to request genomic coordinates for comparison against existing databases.

In some research settings, an annotation, known as “Best Effect” annotation, may be given. This annotation considers all the transcriptional outcomes of all known transcripts at that location and reports the most deleterious (McLaren et al. 2016). As an example, if 15 known transcripts were identified for a single gene, and these were anticipated to result in 10 different transcriptional outcomes including intronic, missense, and splice site variants, the Best Effect annotation would report the splice site alteration because that would most affect translation. As the canonical transcript is sometimes chosen arbitrarily, and different transcripts of the same gene are transcribed in different organs, it is not currently clear which transcript is active at any given time in the tumor, so reporting the most severe effect may be desirable (Barrera et al. 2008; Singer et al. 2008; Wang et al. 2008; Gupta et al. 2010). The Best Effect annotation will frequently not match listed alterations in germline databases and genomic coordinates should be retrieved from the testing laboratory.

Tumor Heterogeneity

Human cancer cells in a given tumor are known to display differences in cellular morphology, metabolism, motility, proliferation, and metastatic potential (Fidler and Hart 1982; Heppner 1984; Nicolson 1984; Dick 2008). This extends to gene alteration and expression. Each tumor is a small universe controlled with Darwinian evolution of tumorigenesis, in which a high level of cell divisions creates a perfect environment for multiple mutants, potentially acting in response to a variety of intratumor and extraorganism pressures. As the analyzed tumor in most cases represents just a small locus of this universe, the analysis may not capture the germline alteration, either because of loss through CNVs or reversion, and therefore the germline alteration

does not appear on the report (Cheng et al. 2015; Cortes-Ciriano et al. 2017; Bailey et al. 2018; Knijnenburg et al. 2018).

Allelic Fraction

Allelic fraction, or the percent of the reads identifying the given alteration, has been posited as a method for determination of possible germline result. However, allelic fraction can be biased by multiple factors (Cheng et al. 2017; Knijnenburg et al. 2018). Indel alterations have an allelic fraction that frequently falls outside of anticipated 40%–60% ranges for germline variants (Cheng et al. 2017). If the given sample for analysis has a high level of tumor content, the allelic fraction may be biased by the heterogeneity of the sample itself. If the tumor purity has been compromised and the sample contains a high level of normal cells, the allelic fraction may also be impacted (Bailey et al. 2018). Homopolymer repeat regions may falsely elevate the amount of reads at any given location, therefore causing an imbalance of allelic fraction between the alteration and wild type (Cheng et al. 2017). Additionally, if a sample contains a high level of CNVs, allelic dropout in some cells of either the wild-type allele or altered allele could lead to a misleading allelic fraction that varies widely from the anticipated 50% (Knijnenburg et al. 2018; Sun et al. 2018). Last, reversion is a well-documented phenomenon, and loss of the mutant allele in samples from previously treated disease may mask an underlying germline variant (Domchek 2017; Mayor et al. 2017; Weigelt et al. 2017).

Presence of Founder Variants

The presence of a common founder variant is one of the best predictors that a somatic alteration is germline. Founder variants are well-typified in many genes including the *BRCA1* and *BRCA2* Ashkenazi Jewish founder variants (*BRCA1* c.68_69delAG and c.5266dupC, *BRCA2* c.5946delT) and Eastern European variants in *CHEK2* (c.1100delC). The presence of one of these founder variants in a tumor specimen supports the need for germline analysis (Knijnenburg et al. 2018). Conversely, a com-

mon founder variant may be filtered out of a somatic testing report if the filters on the somatic analysis are such that an alteration with population frequency >1% (typically derived from Gnomad [Karczewski et al. 2019] or ExAc [Lek et al. 2016]) are automatically filtered out (Cheng et al. 2015; Li et al. 2017).

Discordant Tumor Types

Although defining typical alterations within categories of tumors is still underway, many of the genes that increase empiric risk of cancer in certain organs are frequently involved in tumorigenesis in those same organs (Bailey et al. 2018). However, for most genes, their presence as prominent somatic alterations in discordant tumors should be a flag for possible germline involvement. For example, the presence of a *BRCA2* variant in papillary thyroid cancer should be considered for germline analysis. *TP53* and *PTEN* would be notable exceptions to this rule because of their constitutive nature and relatively common somatic association of multiple tumor types. (Bailey et al. 2018) MSK-IMPACT has found that among tumor paired normal samples with clinically actionable germline alterations, discordance between tumor type and cancer susceptibility gene was identified in 60% of individuals (Cheng et al. 2017). This suggests that an alteration identified in a gene not typical to the tumorigenesis process of that organ, particularly one that would be pathogenic in the germline setting, should be considered for germline genetic analysis.

Tumor Mutational Burden

High tumor mutational burden (TMB) is another scenario in which one should consider genetic testing. Although TMB and MSI have a high level of correlation (Huang et al. 2018), they do not assess for the same somatic phenomena. TMB is the number of relative variants within tumor cells versus the “typical,” and MSI refers specifically to the number of repeat expansions of microsatellites of DNA. For tumors primarily driven by the mismatch repair (MMR) genes, high TMB will be detected (Alexandrov et al.

2013; Kim et al. 2013; Cortes-Ciriano et al. 2017; Bailey et al. 2018; Knijnenburg et al. 2018). In cancers known to be associated with the Lynch spectrum (e.g., colon, gastric, uterine, ovarian, urothelial, and kidney), referral to genetics should be considered if a high level of TMB exists (Cortes-Ciriano et al. 2017; Bailey et al. 2018). It is important to note that although the germline variant may be identified in the somatic analysis, allelic frequency may be an unreliable flag because of the hypermutated nature, and allelic loss of the germline alteration may have happened if large numbers of CNVs are present (Cortes-Ciriano et al. 2017; Bailey et al. 2018).

Variant Classification

Joint Consensus Recommendations for the Standards and Guidelines in the Interpretation and Reporting of Sequence Variants is made by the Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP). Classification based on their recommendation is based on a four-tier system (Table 2): tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants of unknown clinical significance; and tier IV, variants deemed benign or likely benign (Li et al. 2017). Unlike germline variants, in which measurable increased risk of cancer over a lifetime is an indicator of pathogenicity, somatic variants are interpreted based on their impact on clinical care. Therefore, those alterations that predict sensitivity, resistance, or toxicity to a specific therapy can be targeted as part of standard or investigational therapy (i.e., clinical trials). Those alterations that affect prognosis or diagnosis will be tiered in the highest tier. Alterations known to be pathogenic in the germline setting that do not fit the above criteria may therefore be placed in a lower tier. As an example, if a breast cancer patient were to carry a germline *EGFR* T790M variant, this may not be classified as a tier I alteration, as it has no bearing on the patient’s breast cancer treatment. It is important to remember that the primary goal of germline

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Table 2. Somatic variant classification system

Tier I	Variants of strong clinical significance	Level A evidence	FDA-approved therapy
		Level B evidence	Included in professional guidelines
Tier II	Variants of potential clinical significance	Level C evidence	Well-powered studies with consensus from leaders in the field
		Level D evidence	FDA-approved therapies for different tumor types or investigational therapies
Tier III	Variants of unknown clinical significance	Level C evidence	Multiple small published studies with some consensus
		Level D evidence	Preclinical trials or a few case reports without consensus
Tier IV	Benign or likely benign variants	Level C evidence	Not observed at a significant allele frequency in the general or specific subpopulation databases, or pan-cancer or tumor-specific variant databases
		Level D evidence	No convincing published evidence of cancer association
Tier IV	Benign or likely benign variants	Level C evidence	Observed at significant allele frequency in the general or specific subpopulation databases
		Level D evidence	No existing published evidence of cancer association

annotation is to identify alterations causing measurable changes in risk at the whole-body level. Annotation in the somatic setting, however, is used to identify those alterations that create a clinically actionable change in that type of tumor in that particular organ. This classification is by necessity not static and relies on the currently available technologies, therapeutic targets, and clinical trials. Although the joint consensus recommendations do indicate that those variants at high likelihood of being germline, either because of identification in a paired normal sample or because of other features (i.e., 50% allelic fraction in alterations identified as pathogenic in germline databases, known founder variants), should be classified as tier 1, germline alterations can be hard to detect or have possible ambiguous annotation and therefore may be relegated to a lower tier (Li et al. 2017).

In contrast, guidelines for germline analysis are put forth by the American College of Medical Genetics (ACMG). They include a five-basket system that classifies an alteration as Pathogenic (P), Likely Pathogenic (LP), Variant of Uncertain Significance (VUS), Likely Benign (LB), and Benign (B). Variants are scored with two scales; one “Evidence of Benign Impact” and the other “Evidence of Pathogenicity”. The lines of evidence that support pathogenicity are classified as Very Strong, Strong, Moderate, Supporting. The lines of evidence that support benign impacts are classified as Stand Alone,

Strong, and Supporting. Set combinations of this classification correlate to the variant basket and lead to the final classification (Richards et al. 2015).

Case 1

A 37-year-old man presented to the emergency room with rectal bleeding. Colonoscopy identified a 3-cm mass in the cecum. Adenocarcinoma is confirmed by biopsy. He underwent a right hemicolectomy. Immunohistochemistry was performed with all four proteins (MLH1, MSH2, MSH6 and PMS2) present and MSI detected at 0:10 markers. On somatic analysis, the tumor showed a high level of TMB. The specimen was 60% tumor. Two *MSH6* tier III variants were detected: c.3938_3939insTCAAAAGG GACATAGAAAA (p.A1320Sfs*5) in 6% of reads and c.1309C>G (p.H437D) in 5% of reads. Germline large gene panel analysis was performed, and the patient was found to carry the *MSH6* p.A1320Sfs*5 pathogenic variant.

This case illustrates the sometimes-increased sensitivity of somatic analysis. The particular variant identified is a well-known, pathogenic, truncating variant that causes loss of 41 terminal amino acids, leading to a non-functioning protein that can escape nonsense mediated decay. Therefore, a false negative was identified on IHC. An important note is that the allelic fraction in this sample was low, likely

driven down by the overall hypermutability of this tumor. These identified somatic alterations were classified as tier III variants based on the low allelic frequency and lack of actionability in regard to treatment (immunotherapy was not available at the time of this diagnosis).

Germline genetic testing would have been indicated for this patient based on age at diagnosis. Additionally, high TMB in absence of loss of IHC could indicate a polymerase gene variant (i.e., *POLE*, *POLD1*), and these genes were included when the patient's germline testing was ordered.

CLONAL HEMATOPOEISIS OF INDETERMINATE POTENTIAL (CHIP)

The advent of NGS technologies brought an increased sensitivity to detecting genetic variants in blood cells at low allele frequencies. Sanger sequencing can detect mosaic alleles at a 15%–20% allele frequency (Rohlin et al. 2009), whereas NGS can detect low-level mosaicism down to a 2% allele frequency or lower (Li and Stoneking 2012). Variants found at low allele frequencies can suggest the presence of mosaicism acquired during embryogenesis or clonal hematopoiesis (CH) (i.e., the expansions of cells with somatic variants) (Steensma et al. 2015). Clonal hematopoiesis of indeterminate potential (CHIP) generally refers to CH that involves driver mutations associated with myelodysplastic syndromes, such as *DNMT3A*, *ASXL1*, or *TET2* (Steensma 2018). Patients with CHIP have an increased risk of progressing to a heme malignancy, of ~0.5%–1% per year (Sperling et al. 2017), and CHIP has also been associated with acute cardiovascular events (Jaiswal et al. 2017). CHIP may also be a risk factor in the setting of bone marrow transplant as there have been cases of donor cell leukemia's arising from CHIP (Gondek et al. 2016), but other studies have suggested CHIP has no impact on overall survival of donor recipients (Frick et al. 2019). CH can be found in >10% of the population over age 65 (Jaiswal et al. 2014), representing significant implications for clinicians interpreting germline genetic testing results. How to follow patients with age-related CH or CHIP, given the poten-

tial impact on risks for cardiovascular events and heme malignancies, has not been determined.

CELL-FREE TUMOR DNA (ctDNA)

Cell-free DNA (cfDNA) is free-floating DNA in many different body fluids including plasma, saliva, lymph, breast milk, bile, urine, and spinal fluid (Thierry et al. 2016; Wan et al. 2017). Cell-free tumor DNA (ctDNA) refers specifically to short pieces of DNA derived from a tumor and appears in these fluids. ctDNA develops from either cellular breakdown, apoptosis and necrosis, or through active release mechanisms, encapsulated in vesicles, or associated with protein complexes (Jahr et al. 2001; Rykova et al. 2012). Although cfDNA is common even in healthy people, patients with cancer have higher blood levels of cfDNA (Leon et al. 1977; Stroun et al. 1987; Schwarzenbach et al. 2011). The average proportion of mutated DNA in plasma is very low, 0.4% in even advanced cancers (Barbany et al. 2019). Analysis of ctDNA is known as liquid biopsy. Liquid biopsy is performed using NGS to capture tumor-specific genetic variants. Data suggests that the length of ctDNA is shorter than naturally occurring cfDNA, so liquid biopsy utilizes size selection for shorter DNA fragment lengths to increase sensitivity of the analysis (Jiang et al. 2015; Underhill et al. 2016; Hellwig et al. 2018). Ideally, these represent a better summary of the heterogeneity of the genomic landscape of a patient's tumor than perhaps a localized biopsy would. This technology could also increase early stage detection and provide genetic information about a tumor in a hard to biopsy location (Hiemcke-Jiwa et al. 2018; Zill et al. 2018).

A large-scale study in 21,807 patients with advanced cancers in more than 50 cancer types utilized a panel of 70 defined cancer genes to determine the percentage of alterations in these patients. Somatic alterations were identified in 85% of the patients overall, with a range of 51% to 93% represented across various tumor types (Zill et al. 2018). The posited applications of ctDNA include identification of disease progression (i.e., ductal carcinoma in situ to invasive ductal breast cancer), cancer screening in

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high-risk populations (i.e., prostate cancer in men over 70), and distinguishing benign from malignant disease (Barbany et al. 2019). In addition to single-gene variants, it is possible that large CNVs or chromosomal disorders may be identified (Barbany et al. 2019). Additionally, there are a number of clinical trials using liquid biopsy in known variant carriers to detect early onset of tumors (Barbany et al. 2019).

IDENTIFYING GERMLINE VARIANTS THROUGH SOMATIC ANALYSIS

It is important to emphasize that there are no consensus recommendations for assessing which somatic variants should be confirmed with germline hereditary cancer testing other than recent National Comprehensive Cancer Network (NCCN) guidelines that recommend testing for all somatic pathogenic variants found in *BRCA1* or *BRCA2* (NCCN 3.2019). Insurance coverage for germline confirmatory testing varies. Recommendations for such testing are likely to evolve significantly as our understanding of this testing and technologies evolve. However, here we hope to begin the development of a framework for assessment based on the information previously reviewed (see Fig. 1).

Step 1. Assess all cases for clinical testing criteria for hereditary cancer risk based on personal and family history. Given the potential for germline variants to be lost in somatic tumor testing, even cases with no suggestive somatic variants should be offered germline testing based on appropriate clinical indications.

Step 2. Determine if tumor testing shows high TMB or MSI-high features (and *BRAF*-negative if colorectal tumor). These features are highly suggestive of variants in the genes associated with Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) as well as *POLE* and *POLD1*, and subsequent germline testing should be done that includes these genes, at a minimum. Although these genes are typically included in somatic testing, the genes involve difficult to sequence regions, such as the pseudogene region of *PMS2*, which may be excluded from somatic analysis.

Step 3. Ensure that the somatic tumor result has not filtered out germline findings. If so, any decision to proceed with germline testing will be based solely on personal and family history guidelines or other germline-variant prediction models.

Step 4. Review somatic results for common founder variants. These are highly likely to be confirmed in the germline. Examples include the three common variants in the *BRCA1* and *BRCA2* genes associated with Jewish ancestry. It is less clear if testing is appropriate for moderate penetrance founder variants, although many of them impact cancer screening recommendations. These could include monoallelic variants in *MUTYH* (c.Y179C and c.G396D), *APC* c.I1307K, *NBN* c.657del5, and *CHEK2* c.1100delc.

Step 5. At this point, the reviewer may be left with several variants in genes known to be associated with hereditary cancer risk and can begin the process of separating variants using the five-basket system: Benign (B), Likely Benign (LB), of Uncertain Significance (VUS), Pathogenic (P), and Likely Pathogenic (LP). Some variants will simply be noted as “detected” or similar, and further investigation may be necessary (Table 3). Variants confirmed to be B, LB, or VUS in the germline setting will generally not need germline testing unless other clinical indications are met.

Step 6. Some genes have a higher likelihood of being confirmed in the germline than others. NCCN now recommends confirmatory germline testing for all pathogenic and likely pathogenic variants in the *BRCA1* and *BRCA2* genes (NCCN 3.2019). Based on data from paired somatic/germline testing programs, other genes with a significant (>20%) chance of testing positive in the germline when seen in somatic tissue include the Lynch genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) and *PALB2* (Meric-Bernstam et al. 2016). A reasonable argument could be made to proceed with germline testing that includes pathogenic or likely pathogenic somatic variants in any of these genes, but there is limited infor-

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*B = Benign, LB = Likely Benign, VUS = Variant of Uncertain Significance, LP = Likely Pathogenic, P = Pathogenic.

Figure 1. Flowchart to assess somatic results for potential germline testing.

mation regarding incidence of moderate-risk genes, such as *ATM*, *CHEK2*, *RAD51C*, and others. The provider may wish to consider the potential clinical impact of a pathogenic variant for various genes (Table 4).

Step 7. Conversely, many genes common in tumor cells are only rarely confirmed in the germline. These include, but are not limited to, *APC*, *TP53*, *MEN1*, *NF2*, *PTEN*, *RET*, *STK11*, and *VHL* (Meric-Bernstam et al.

2016). The syndromes associated with these genes tend to have distinctive phenotypes. Although clinical assessment is not always a perfect predictor of germline variants, given the low overall proportion of germline variants in these genes, it may be appropriate to proceed with testing of P/LP variants only when there are additional clinical indications. Sometimes, the nonquantifiable “clinical judgment” may come into play.

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Table 3. Resources to help assess somatic variants for germline testing

Transcript determination	Ensembl genome browser	useast.ensembl.org/index.html
	UCSC Genome Browser	www.genome.ucsc.edu
	NCBI Genome	www.ncbi.nlm.nih.gov/genome
	RefSeqGene	www.ncbi.nlm.nih.gov/refseq/rsg
Somatic variant classification	My Cancer Genome	www.mycancergenome.org
	cBioPortal, Memorial Sloan Kettering	www.cbioportal.org
	IARC TP53 mutation database	p53.iarc.fr
	International Cancer Genome Consortium	www.icgc.org
Germline variant classification	Catalog of Somatic Mutations in Cancer (COSMIC)	cancer.sanger.ac.uk/cosmic
	Clinvar	www.ncbi.nlm.nih.gov/clinvar
	Clinvite	clinvite.invitae.com
	International Society for Gastrointestinal Hereditary Tumors (InSiGHT)	www.insight-database.org
Gene tumor frequencies	Human Gene Mutation Database (HGMD)	www.hgmd.cf.ac.uk/ac/index.php
	Leiden Open Variant Database	www.lovd.nl
	cBio Portal	www.cbioportal.org
	COSMIC	cancer.sanger.ac.uk/cosmic
Germline population frequencies	ExAC	exac.broadinstitute.org/
	Genome Aggregation Database (gnomAD)	gnomad.broadinstitute.org/
	1000 Genomes	browser.1000genomes.org
	Exome variant server	evs.gs.washington.edu/EVS
Gene association	dbSNP	www.ncbi.nlm.nih.gov/snp
	dbVar	www.ncbi.nlm.nih.gov/dbvar
	GeneReviews	www.ncbi.nlm.nih.gov/books/NBK1116
	GeneCards	www.genecards.org
	Genetics Home Reference	www.ghr.nlm.nih.gov
	Online Medelian Inheritance in Man (OMIM)	www.omim.org

Step 8. Debatable, other genes should be considered for confirmatory germline testing. The ACMG has developed a list of 25 cancer genes (among others) representing highly penetrant genetic disorders in an attempt to reduce the morbidity and mortality associated with these genes (Kalia et al. 2017) and this list may serve as a baseline for some clinicians. Many of the publications examining results from paired somatic and germline testing to date have not included moderate-penetrance genes such as *CHEK2* and *ATM*, yet pathogenic variants from these genes have the potential to affect cancer risk screening, given recommendations from NCCN and others (NCCN 1.2018; NCCN 3.2019). See Table 4.

As a final step, there will need to be a process developed to follow up with the ordering provider or the patient in order to ensure that co-

ordination of germline testing is appropriately facilitated.

Case 2

A 54-year-old man was recently diagnosed with Stage IV gastric adenocarcinoma with signet ring cells, also known as diffuse gastric cancer (DGC). After progressing through several lines of therapy, he underwent testing with a commercial multiplatform, solid tumor biomarker analysis. The tumor was found to be Her2/Neu-negative by IHC and MSI-stable. Two somatic variants were noted, a pathogenic variant in *TP53* and a variant of uncertain significance in *CDH1*. The patient was referred to a genetic counselor for consideration of germline genetic testing.

The genetic counselor involved with this case noted that ~12% of gastric cancers will

Table 4. Hereditary cancer risk genes and screening implications

Hereditary cancer risk genes	ACMG ^a	Screening guidelines	Hereditary cancer risk genes	ACMG ^a	Screening guidelines
<i>APC</i>	✓	NCCN- GI	<i>NF1</i>		NCCN- HBOC
<i>APC I1307K</i>		NCCN- GI	<i>NF2</i>	✓	Evans et al. 2005
<i>ATM</i>		NCCN- HBOC	<i>NTHL1</i> (biallelic only)		NCCN- GI
<i>AXIN2</i>		NCCN- GI	<i>PALB2</i>		NCCN- HBOC
<i>BAP1</i>		Rai et al. 2016	<i>POLD1</i>		NCCN- GI
<i>BARD1</i>		N/a	<i>POLE</i>		NCCN- GI
<i>BMPR1A</i>	✓	NCCN- GI	<i>PTEN</i>	✓	NCCN- HBOC
<i>BRCA1</i>	✓	NCCN- HBOC	<i>RAD50</i>		N/a
<i>BRCA2</i>	✓	NCCN- HBOC	<i>RAD51C</i>		NCCN- HBOC
<i>BRIP1</i>		NCCN- HBOC	<i>RAD51D</i>		NCCN- HBOC
<i>CDH1</i>		NCCN- HBOC	<i>RB1</i>	✓	Skalet et al. 2018
<i>CDKN2A</i>		Rossi et al. 2019	<i>RET</i>	✓	Kloos et al. 2009
<i>CHEK2^b</i>		NCCN- HBOC	<i>SDHA</i>	✓	Lenders et al. 2014
<i>CTNNA1</i>		N/a	<i>SDHB</i>	✓	
<i>DICER1</i>		Schultz et al. 2018	<i>SDHC</i>	✓	
<i>GREM1</i>		NCCN- GI	<i>SDHD</i>	✓	
<i>HOXB13</i>		Giri et al. 2018	<i>SMAD4</i>	✓	NCCN- GI
<i>MEN1</i>	✓	Thakker et al. 2012	<i>STK11</i>	✓	NCCN- HBOC
Lynch syndrome/MMR genes (<i>MLH1</i> , <i>MSH2</i> , <i>MSH6</i> , <i>PMS2</i> , <i>EPCAM</i>)	✓	NCCN- GI	<i>TP53</i>	✓	NCCN- HBOC
<i>MSH3</i> (biallelic only)		NCCN- GI	<i>TSC1</i>	✓	Krueger et al. 2013
<i>MUTYH</i> (monoallelic)		NCCN- GI	<i>TSC2</i>	✓	
<i>MUTYH</i> (biallelic)	✓	NCCN- GI	<i>VHL</i>	✓	Poulsen et al. 2010
<i>NBN</i>		NCCN- HBOC	<i>WT1</i>	✓	Lee et al. 2016

Genetic/Familial High-Risk Assessment-Gastrointestinal (NCCN-GI): Colorectal. Version 1.2018

Genetic/Familial High-Risk Assessment-Hereditary Breast and Ovarian Cancer (NCCN-HBOC): Breast and Ovarian. Version 3.2019

^aACMG Secondary Finding Genes (Kalia et al. 2017).^bLow-penetrance variants, such as S428F and I157T, have unclear clinical implications.

have somatic variants in *CDH1*, and there is limited data as to the proportion of somatic variants that are later confirmed in the germline. The reported somatic variant, c.D257Y, was classified as a variant of uncertain significance in the germline. In hereditary diffuse gastric cancer (HDGC) syndrome the average age of onset of DGC is 38 yr (range: 14–69 yr) and testing is recommended for anyone diagnosed at age 40 or younger. When diagnosed at older ages, additional family history of gastric cancer or lobular breast cancer is part of testing criteria (van der Post et al. 2015). Although the patient did not have known breast or gastric cancer in his family, there was some question about his father

having esophageal versus gastric cancer. Whereas the counselor and patient discussed the low likelihood of finding an actionable variant, the patient was interested in pursuing testing and willing to pay out-of-pocket costs, as he did not meet clinical criteria for testing. One potential benefit of testing would be to have the VUS status confirmed by a clinical laboratory, which would allow for future reclassification updates. Testing was pursued through a multigene cancer panel from a commercial laboratory.

Germline results showed a new pathogenic variant in *CDH1*, called deletion exons 14–16, which had not been previously identified in the somatic test report. This new variant was noted

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to involve a large deletion that may not have been detectable through the somatic testing laboratory processes. The discovery of this germline variant has allowed for cascade testing of several family members.

This case illustrates the limitations of somatic genetic testing in regards to certain types of variants and also highlights some of the remaining challenges when determining which somatic tumor findings justify follow-up germline testing.

DISCUSSION

Although this review speaks to the current state of practice, many new changes are on the horizon. It is likely that most somatic analysis will become paired with germline analysis, and the ambiguity surrounding whether a somatic gene change is also present in the germline will no longer exist. These changes will hopefully lead to higher rates of insurance coverage for comprehensive germline analysis in cancer patients, likely extending testing to cancer patients that do not fit current guidelines for analysis. Additionally, somatic analysis has already led to increased understanding of how genes typically classified as “cardiac” or “general genetics” may contribute to cancer risk (i.e., *NOTCH1*, Rasopathies, *RECQL1*). The continued identification of crossover risks may lead to greater fluidity between the traditionally siloed specialties within genetics.

Significant limitations exist at this time to this summary of the science. Although it is known that methylation, structural variants, and variation in the introns and promoters are likely involved in tumorigenesis, comprehensive analysis of these areas and types of genomic variation is uncommon, and we do not understand the implication of these alterations. Certainly, as our understanding of these variations deepens, the extent to which we acknowledge the interplay between somatic and germline variants will also broaden. The current data regarding somatic testing is limited by the reliance on sequencing and deletion and duplication of the DNA strand only.

As somatic technologies have primarily been performed in countries of European descent and among well-insured individuals in the United States, there is a significant lack of data about tumor profiles in individuals from non-White and Hispanic racial and ethnic groups. Until genomic research, both germline and somatic, is intentionally designed to capture genomic data from individuals that represent the global population, its applicability to understanding and treating cancer is hampered. There are likely many genetic nuances in each population that affect prognosis, response, baseline risk, and recurrence risk, and the current extrapolation of existing research to populations not included in the source data is limiting.

Further research will be needed to understand how other genomic changes in the germline (methylation, variable expression) effect tumorigenesis, how the tumorigenesis process may differ in racial and ethnic communities outside of European ancestry and how best, or if at all, to silo identified variants into areas of specialty.

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