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Point/Counterpoint

Immunohistochemistry versus Microsatellite Instability Testing for Screening Colorectal Cancer Patients at Risk for Hereditary Nonpolyposis Colorectal Cancer Syndrome

Part II. The Utility of Microsatellite Instability Testing

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Germline mutations in the mismatch repair genes mutL homolog 1 (*MLH1*) and mutS homolog 2 (*MSH2*), *MSH6*, and postmeiotic segregation increased 2 (*PMS2*) lead to the development of hereditary nonpolyposis colorectal cancer (HNPCC). Diagnosis of HNPCC relies on the compilation of a thorough family history of cancer, documentation of pathological findings, tumor testing for microsatellite instability (MSI) and immunohistochemistry (IHC), and germline mutation analysis of the suspected genes. As a hallmark of HNPCC, microsatellite instability is widely accepted as a primary method for identifying individuals at risk for HNPCC. It serves as an excellent, easy-to-evaluate marker of mismatch repair deficiency. Recent improvements in MSI testing have significantly enhanced the accuracy and reduced its cost. Proficiency testing for MSI is available, and laboratory-to-laboratory reproducibility of such testing can be easily evaluated. In addition, the combination of microsatellite instability testing, *MLH1* promoter methylation analysis, and *BRAF* (V600E) mutation analysis can distinguish a sporadic colorectal cancer from one associated with HNPCC, helping to avoid costly molecular genetic testing for germline mutations in mismatch repair genes. In this article, we discuss the development of MSI markers used for HNPCC screening and focus on the advantages and disadvantages of MSI testing in screening for HNPCC patients. We conclude that MSI is as sensitive and specific as IHC, given its excellent reproducibility and its potential capability to indicate mutations not be detected by IHC. MSI has been used and will continue to prevail as the primary screening tool for identifying HNPCC patients. ***(J Mol Diagn 2008, 10:301–307; DOI: 10.2353/jmoldx.2008.080062)***

The diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC) at the molecular level relies on the presence of a deleterious germline mutation in one of the mismatch repair (MMR) genes. Because cancer morbidity and mortality can be dramatically reduced by colonoscopic screening of individuals with the HNPCC syndrome and by prophylactic surgeries, molecular screening of colorectal cancer patients for HNPCC is now feasible.[1–4] The challenge is to establish a strategy that is able to screen effectively for HNPCC. Microsatellite instability (MSI) in colorectal cancer was discovered in 1993 and was subsequently found to be present in colon cancer tissue from most HNPCC patients.[5–8]

Genotyping for microsatellite instability was initially used to screen for HNPCC,[1,3] while immunohistochemistry (IHC) analysis of the MMR proteins has been more recently proposed as an alternative method for screening HNPCC.[2] Two recent studies have indicated that microsatellite instability testing and immunohistochemistry are both highly effective strategies for selecting patients for molecular genetic testing (germline mutation analysis).[2,9] However, it is unclear which approach should be used as the primary method for screening HNPCC. Here, we summarize both the early and more recent literature data on the use of MSI, discuss the

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molecular basis of microsatellite instability in MMR-deficient tumors, and outline the advantages and limitations of this methodology. Our analysis indicates that given several merits of MSI that IHC does not have (see Advantages of MSI, below), MSI is an excellent, easy to use marker for identifying HNPCC. Therefore, it is important that clinicians are aware of the pros and cons of these two tests as both are widely used in screening HNPCC cases.

Literature Review

Microsatellite Instability

Microsatellites are short, tandemly repeated DNA sequences of 1 to 6 bases scattered throughout the human genome;[10,11] they can be affected by a form of genomic instability called microsatellite instability.[5,6,8,12] MSI is a change in length of a microsatellite allele due to either insertion or deletion of repeating units during DNA replication and failure of the DNA mismatch repair system to correct these errors. MSI analysis has been used as a screening method to identify HNPCC patients and a subgroup of colorectal cancer patients for further genetic testing.

The DNA Mismatch Repair System and HNPCC

DNA MMR is an effective post-replication mechanism. Most errors that occur during DNA replication are immediately corrected by the 3′ to 5′ exonuclease activity of DNA polymerase. It is estimated that 99.9% of the mutations that escape the proofreading activity of DNA polymerase (DNA polymerase slippage) are repaired by the DNA MMR system, particularly single-bp mismatches and "loop outs" of unpaired bases.[13] The replication machinery slips more frequently on repetitive sequences than on non-repetitive sequences, so microsatellite instability occurs in the repetitive sequences in MMR-deficient cells. The causes of MMR defects are: i) germline mutations in any one of the five DNA MMR genes—mutS homolog 2 (*MSH2*), mutL homolog 1 (*MLH1*), *MSH6*, and, infrequently, postmeiotic segregation increased 2 or 1 (*PMS2* or *PMS1*), causing HNPCC[14]; and ii) somatic inactivation of *MLH1* caused by promoter hypermethylation in approximately 15% of sporadic colorectal cancer.[5,8,15]

In MMR-deficient cells, genes that contain a microsatellite in their coding regions are more prone to frameshift mutations. Mutations in key genes that regulate cell growth and apoptosis ultimately lead to dysregulated cell proliferation and/or cell death, which further speeds the evolution of colorectal cancer.[16] One example is the well studied frameshift mutations in the *TGF-βRII* gene, which commonly occurs in colorectal cancer but not in endometrial cancer. In most colorectal cancers, the polyadenine tract mutations affect both alleles of *TGF-βRII*, suggesting that *TGF-βRII* functions as a tumor suppressor during colorectal cancer development and is a critical target of inactivation in mismatch repair-deficient tumors.[17–19] Similar frameshift mutations in coding microsatellites also occur in other genes involved in growth control and apoptosis (*TCF4*, *IGFIIR*, *BAX*, and *RIZ*), as well as in genes involved in DNA mismatch repair itself (*MSH6*, *MSH3*, and *MSH2*).[14]

MSI as a Marker for HNPCC Screening

The original (1997) Bethesda guidelines[20,21] proposed a panel of five microsatellite markers for the uniform analysis of MSI in HNPCC. This panel, which is referred to as the Bethesda panel, included two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123, and D17S250) repeats. Samples with instability in two or more of these markers are defined as MSI-High (MSI-H), whereas those with one unstable marker are designated as MSI-Low (MSI-L). Samples with no detectable alterations are MSI-stable (MSS). Because mononucleotide markers appear to be more sensitive than dinucleotide markers for the detection of MSI-H, limitations in the original panel resulting from inclusion of dinucleotide repeats were addressed at a 2002 National Cancer Institute workshop, and revised recommendations for MSI detection were proposed. The revision mainly recommends testing a secondary panel of mononucleotide markers, such as BAT-40, to exclude MSI-L in cases in which only the dinucleotide repeats are mutated.[22] According to the revised Bethesda guidelines, strategies based on MSI testing were effective in identifying *MLH1/MSH2* mutation carriers (sensitivity 81.8% and specificity 98.0%).[9]

Advantages of MSI

Microsatellite Instability Serves as an Excellent, Easy-to-Evaluate Marker of MMR Deficiency, and Recent Improvements in MSI Testing Significantly Enhance Accuracy and Reduce Cost

A hallmark of tumors in HNPCC is microsatellite instability. Typically half or more of all microsatellites have mutations (contraction or elongation) in the tumor cells; therefore, microsatellite instability serves as an excellent, easy-to-evaluate marker of mismatch repair deficiency. Since both HNPCC and MSI are caused by MMR defects, MSI can be used as a surrogate marker of HNPCC and has been widely accepted as a primary method for identifying individuals at risk for HNPCC.

As mentioned under Literature Review, a recent follow-up NCI workshop recognized the limitations of the original Bethesda panel[20,21] due to the inclusion of dinucleotide repeats, which are less sensitive and less specific than mononucleotide repeats for identification of cancers with MMR deficiency.[22] To improve the accuracy of MSI testing using the Bethesda panel of MSI markers, a panel of five mononucleotide markers was developed and incorporated into a multiplex fluorescence assay: the Promega (Madison, WI) MSI Analysis System.[23] These mononucleotide repeat markers are quasi-monomorphic; that is, almost all individuals are homozygous for the

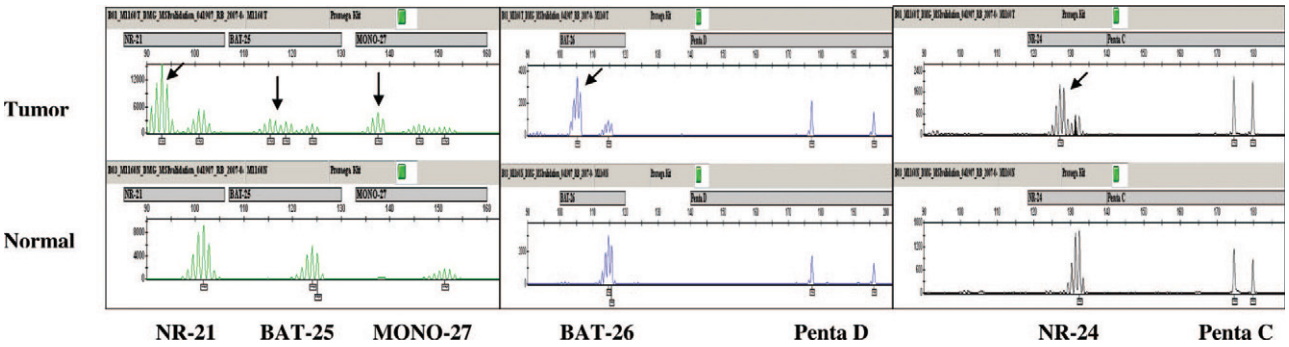

Figure 1. Representative electropherograms of the Promega MSI Analysis System (Version 1.1) generated using GeneMapper 3.7 Analysis Software. The shifted alleles are indicated by an **arrow**. Green: electropherogram showing the peaks of 2′,7′-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE)-labeled loci, NR-21, BAT-25, and MONO-27. Blue: electropherogram showing peaks of the fluorescein-labeled loci, BAT-26 and Penta D. Black: electropherogram showing the peaks of tetramethyl rhodamine (TMR)-labeled loci, NR-24 and Penta C.

same common allele for a given marker. The use of monomorphic markers simplifies data interpretation. The added pentanucleotide repeat markers ensure that the tumor and matching normal specimen are from the same individual.[23,24] The Promega system can help resolve cases of MSI-L into either MSI-H or MSS.[25] The representative electropherograms of the Promega MSI Analysis System are shown in Figure 1. The microsatellite markers included in the Bethesda panel[20–22] and the Promega MSI Analysis System,[25,26] as well as other commonly used MSI markers, are summarized in Table 1.

Since the Promega MSI Analysis System utilizes a multiplex fluorescence assay, PCR for all five mononucleotide markers and two pentanucleotide nucleotide markers can be performed in a single reaction. The size of the amplified products can be easily visualized using capillary electrophoresis. The cost of MSI testing is significantly reduced. In addition, in situations where the availability of representative tumor sections is limited, MSI testing can be done on one tumor section, whereas at least four tumor sections are required for IHC.

Proficiency Testing for MSI Is Available and the Reproducibility of MSI Testing is Close to 100%

The most common type of quality control in which clinical laboratories participate is proficiency testing, in which testing is performed along with routine laboratory work. Proficiency testing is a method of externally validating the accuracy of laboratory performance by testing samples and comparing results of all participating laboratories. To comply with Clinical Laboratory Improvement Amendments of 1988, laboratories performing moderate and high complexity tests must be enrolled in regulatory proficiency testing for their particular specialties/subspecialties of testing. A convenient way to accomplish this is by subscribing to a proficiency testing program that monitors those analytes. In the United States, proficiency testing for MSI is provided by the College of American Pathologists (CAP).

In the most recent CAP Proficiency Survey for microsatellite instability testing (2007 MSI-B), 71 laboratories were enrolled in this survey. The participant summary

Table 1. The Loci Information for Commonly Used MSI Markers

Microsatellite marker	Repeat type	Chromosomal location (gene near marker/GenBank number)	Repeat motif*[24,52]	Bethesda panel[20–22]	Promega kit[24,25]
BAT-25	Mononucleotide	4q12 (*c-kit,* intron 16)	TTTT.T.TTTT.$(T)_7$.A$(T)_{25}$	X	X
BAT-26	Mononucleotide	2p16.3-p21 (*hMSH2* gene, intron 5)	$(T)_5$ . . . . . $(A)_{26}$	X	X
NR-21	Mononucleotide	14q11.2 (*SLC7A8,* 5′UTR)	$(T)_{21}$		X
NR-24	Mononucleotide	2q11.2 (*ZNF2*, 3′UTR)	$(T)_{24}$		X
MONO-27	Mononucleotide	2p21 (*MAP4K3*, intron 13)	$(A)_{27}$		X
BAT-40	Mononucleotide	1p13.1 (*HSD3B2*)	TTTT.TT . . $(T)_7$ . . . . TTTT.$(T)_{40}$		
D2S123	Dinucleotide	2p16 (*MSH2*)	$(CA)_{13}$TA$(CA)_{15}$(T/GA)$_7$	X	
D5S346	Dinucleotide	5q21/22 (*APC*)	$(CA)_{26}$	X	
D17S250	Dinucleotide	17q11.2-q12 (*BRCA1*)	$(TA)_7$ . . . . . $(CA)_{24}$	X	
Penta C	Pentanucleotide	21q22.3 (AL138752)	$(AAAAG)_{3–15}$		X
Penta D	Pentanucleotide	9p12-13.3 (AC003656)	$(AAAAG)_{2–17}$		X
MYCL1	Complex	1p34.3 (*MYCL1*)	GAAAA$(GAAA)_2$TAAA$(A/G)_{10}$ GAAAGA$(GAAA)_{14}$ GAAA $(GAAAA)_8$GAAAAA$(GAAAA)_2$		

*Non-repetitive nucleotides are indicated as dots.

provided evidence that mononucleotide markers have higher specificity (ie, a lower false positive rate) for instability than dinucleotide markers. CAP also provided a detailed summary on several clinically important issues such as the number and types of markers used, methods used to perform the assay, and definition of MSI-H and MSI-L phenotypes. This information is valuable to clinical laboratories that are currently offering this test as well as to those that are planning to launch this test. Thirty-four of the 53 laboratories reported performing IHC together with MSI, whereas 19 of 53 laboratories did not perform IHC testing. With two recent CAP Proficiency Surveys combined (2007 MSI-A and MSI-B), 101 of 103 laboratories (98%) reported the same results, indicating that the reproducibility of MSI testing can be evaluated and, more importantly, that it is satisfactorily high.

In terms of quality control and interpretation of MSI testing in clinical settings, studies from a six-center consortium indicated that optimal PCR quality is essential to getting interpretable results. In most cases, the shifted PCR products from all five mononucleotide markers were smaller in size than the germline allele, as deletion in polyA sequences is much more common than insertions.[27,28] It has been suggested that a larger allele size does not correlate with loss of mismatch repair gene expression by immunostaining and overall tumor phenotype. Forcing a call tended to bias toward increased numbers of MSI-L or MSI-H cases. Duplicate readings (scoring MSI results independently by two people) can help to reduce errors.[29]

MSI Analysis Could Potentially Identify a Tumor That Had Defective DNA MMR But Intact Staining, Perhaps Due to Non-Truncating Missense Alteration

Some disease-causing non-truncating mutations (missense mutations or in-frame insertion/deletion mutations) may not affect the protein translation, stability, and antigenicity; therefore, IHC staining is intact. A common scenario for IHC is to find focal and weak, or ambiguous, MLH1 staining along with absent PMS2 staining; this most likely represents a germline missense mutation in *MLH1*. However, when a missense mutation or an in-frame insertion/deletion mutation in *MLH1* resides outside the MLH1/PMS2 interacting domain, IHC for both MLH1 and PMS2 may be present. Just as occurs with *MLH1*, some *MSH6* missense mutations increase the risk for cancer, but MSH6 protein is present in the tumors by IHC. Under these situations, although MLH1 or MSH6 staining is present, MSI is able to identify a tumor that has MMR deficiency.

MSI Analysis Could Potentially Identify a Tumor That Had Defective DNA MMR Due to Defects in Genes Other than MSH2, MLH1, MSH6, *or* PMS2

MSI is a functional analysis in that mutations that disrupt MMR function lead to microsatellite instability in the tumor cells. Since MMR involves a set of genes including, but not limited to, *MLH1*, *MSH2*, *MSH6*, *PMS2, MSH3*, and *PMS1*, MMR deficiency may be caused by mutations in MMR genes not tested by IHC or in as yet unidentified MMR gene. MSI testing shows positive results in the presence of a mutation that disrupts the normal MMR function, no matter if the mutation resides in a known gene or in an uncharacterized MMR gene. IHC cannot detect such abnormalities since the current testing is limited to the four proteins with available antibodies.

Summary

MSI offers the following advantages that IHC does not: i) unlike IHC, which requires well experienced pathologists, molecular laboratory directors can be easily trained to read MSI results; ii) compared with the IHC staining pattern, which may vary and result in uncertainty in interpretation, the MSI unstable pattern can be easily recognized and highly reproducible; iii) unlike IHC, the sensitivity of which is dependent on its antibody panel, with its satisfactorily high reproducibility and the availability of proficiency testing offered by CAP, the quality control of MSI testing can be easily performed in a clinical laboratory; iv) MSI analysis could potentially identify a tumor that had defective DNA MMR but intact staining possibly due to non-truncating missense alterations or mutations in other MMR proteins not included in the current IHC panel; v) when the availability of representative tumor sections is limited, unlike IHC, which requires at least four tumor sections, only one tumor section with reasonable size of tumor is sufficient for MSI testing.

Limitations of MSI

MSI Is Not Specific for HNPCC

Although microsatellite instability is a hallmark for HNPCC, it is not specific for HNPCC. Microsatellite instability has been demonstrated in 10 to 15% of sporadic colorectal cancers. Deficient mismatch repair is largely attributed to hypermethylation of the 5′ CpG island in the *MLH1* promoter and its consequent transcriptional silencing. The methylation is often, but not invariably, associated with loss of *MLH1* protein.[15] This is a so-called epigenetic change that affects gene function by aberrant methylation of DNA without genetic changes at the DNA sequence level. Epigenetic silencing has been recognized as another pathway that inactivates tumor suppressor genes in cancer.[30]

MLH1 hypermethylation is the cause of the microsatellite instability phenotype in sporadic colorectal cancers. Although the frequency of *MLH1* hypermethylation in tumors with well characterized germline genetic defects in MMR genes was significantly reduced relative to sporadic microsatellite instability tumors, the presence of *MLH1* hypermethylation did not exclude the possibility of germline mutations in mismatch repair genes.[15] Actually, *MLH1* hypermethylation may act as the second hit that

inactivates the wild-type *MLH1* allele in HNPCC tumors in addition to somatic mutations and deletions.[31,32]

Recently, the V600E mutation in *BRAF* was reported to be associated with microsatellite-unstable colorectal tumors.[33–35] *BRAF* (V600E) mutation is frequently present in sporadic colorectal cancers with *MLH1* hypermethylation, but not present in HNPCC. *BRAF* (V600E) mutation was not present in 4/4 MSI-H cell lines with mutated *MLH1* or in 20/20 MSI-H HNPCC tumors.[31] In other studies, *BRAF* (V600E) mutation was absent in 18/18 HNPCC tumors,[36] in 37/37 HNPCC tumors,[37] in 111/111 HNPCC tumors, and in 45/45 cases with abnormal MSH2 immunostaining.[38] Based on these recent studies, it was concluded that the presence of *BRAF* (V600E) mutation argues against the presence of a germline mutation in either the *MLH1* or *MSH2* gene in HNPCC-associated colorectal cancers (specificity ~100%).[31,36–38] Tumors that have the *BRAF* V600E mutation and demonstrate *MLH1* promoter hypermethylation are almost certainly sporadic, whereas tumors that show neither are most likely inherited. This will avoid the fruitless germline sequencing and rearrangement analysis of *MLH1*, which is still costly and labor consuming.

Tumors with Germline Mutations in MSH6 *Tend to Show Lower Levels of MSI*

Although the *MSH6* gene is a component of the DNA MMR machinery, tumors with germline mutations in *MSH6* may not show MSI-H. Instead, they tend to show a lower level of MSI.[39–41] This is the consequence of the partial redundancy of the function of MSH6 and MSH3 proteins.[42,43] The MSH2/MSH6 heterodimer (MutSα) recognizes both base-base and insertion-deletion loops, whereas the MSH2/MSH3 heterodimer (MutSβ) mainly recognizes insertion-deletion mismatches larger than one nucleotide.[42,44] This explains the fact that when MSH6 is mutated, the MSH2/MSH3 dimer is still functioning, so that MSI can be limited to mononucleotide tandem repeats. As a matter of fact, addition of the BAT-40 mononucleotide marker reclassified some MSI-L tumors as MSI-H and some MSS tumors to MSI-L.[41] Since MSI in *MSH6* carriers has been mainly observed at mononucleotide markers,[45] we speculate that when a sufficient number of mononucleotide markers are used for MSI testing, more *MSH6* tumors will be considered as MSI-H rather than MSI-L or MSS. This is an area that awaits further investigation.

The fact that few or no differences in expressivity have been detected between *MLH1* and *MSH2* mutations indicates that both are equally important for MMR. In this regard, mutations in *MSH6* behave quite differently. The so-called "attenuated" type of HNPCC caused by mutations in *MSH6* is characterized by lower penetrance, higher age at onset, and more frequently distally localized colon cancers.[39,46] It is reasonable to speculate that some MSS colon tumors in *MSH6* patients are likely to have been sporadic tumors that did not develop because of MMR deficiency.[41] Therefore, HNPCCs with *MSH6* mutations may not be easily recognized because these features differ from those with mutations in *MLH1* or *MSH2*.

Summary

MSI testing can be seen in both HNPCC tumors with germline mutations in *MLH1* and sporadic colorectal cancers with *MLH1* hypermethylation. However, IHC cannot distinguish these two types of colorectal cancers either. Since mutation in *BRAF* is present in the majority of tumors with hypermethylation of the *MLH1* promoter but, importantly, not in cases with germline *MLH1* mutations, the combination of microsatellite instability testing, *MLH1* hypermethylation, and *BRAF* (V600E) mutation analysis can help distinguish a sporadic colorectal cancer from one associated with HNPCC. As for the *MSH6* mutations, since the frequency of missense mutations is similar to that of truncating mutations and missense mutations in *MSH6* may not alter its antigenicity, some mutations in *MSH6* will be equally missed by IHC. In this regard, *MSH6* mutation analysis should be considered in all patients suspected to have HNPCC. Neither MSI nor IHC should be a definitive selection criterion for *MSH6* mutation analysis.[39]

Conclusion and Perspectives

MSI analysis has been used as a screening test for HNPCC in the original and revised Bethesda guidelines for over 10 years, and we have accumulated a wealth of experience with this test. The recent development of the five-mononucleotide marker system (the Promega MSI Analysis System) has greatly enhanced the accuracy and reduced the cost of MSI testing. Since the Promega system can help resolve cases of MSI-L into either MSI-H or MSS, and MSI-L is a major issue of *MSH6* tumors, we speculate that, with the utility of this new MSI system, MSI testing will be better able to characterize the *MSH6* tumors and assist in the search for germline mutations in *MSH6*. With its satisfactorily high sensitivity, specificity, and reproducibility, MSI offers myriad advantages for HNPCC screening. It is worthwhile to mention that CAP proficiency testing on MSI makes quality control as easy as possible to implement in clinical settings. Therefore, it is recommended that for patients who meet the Bethesda guidelines, the first step would be MSI analysis followed by IHC of all tumors classified as MSI-H.[47]

It is a fact that about half of all families clinically defined as HNPCC do not have mutations in any of the known MMR genes. This is an active research area in which new genes involved in MMR await to be identified and characterized. In this regard, MSI analysis could potentially identify mutations in other MMR genes not included in the current IHC panel. That is, if no mutations are present in the currently well studied MMR genes, a deleterious mutation might be present in hitherto unidentified genes. It is also possible that a mutation is present in the currently known MMR genes, but leads to HNPCC via completely different molecular mechanisms. The recent reports on germline methylation of the *MLH1* or *MSH2* promoter

leading to HNPCC provide examples of these novel mechanisms.[48–51]

To some extent, MSI and IHC are complementary to each other in identifying HNPCC. The issue is that, currently, molecular testing for HNPCC screening is available at academic hospitals and major cancer centers as well as some private diagnostic companies; it is important that we make efforts to extend the current small-scale use of these molecular diagnostic tools and make them available to all clinicians. In order for this effort to be successful, clinicians must become aware of the advantages and disadvantages of these tests.

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