Technology Assessment

Technology Assessment Program

A systematic review of loss-of-heterozygosity based topographic genotyping with PathfinderTG®

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A systematic review of loss-of-heterozygosity based topographic genotyping with PathfinderTG®

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Tufts Evidence-based Practice Center

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None of the investigators has any affiliations or financial involvement related to the material presented in this report.

Peer Reviewers

We wish to acknowledge individuals listed below for their review of this report. This report has been reviewed in draft form by individuals chosen for their expertise and diverse perspectives. The purpose of the review was to provide candid, objective, and critical comments for consideration by the EPC in preparation of the final report. Synthesis of the scientific literature presented here does not necessarily represent the views of individual reviewers.

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Summary

Microscopic (pathologic) analysis of tissue samples is central to the diagnosis and management of patients with malignancy. There are several instances where the morphologic analysis of a tissue specimen is inconclusive and may not be able to inform management decisions (e.g., trying to distinguish a metastatic tumor from a de novo primary tumor), or in some instances trying to distinguish malignant from nonmalignant tissue (e.g., sorting apart glial neoplasms from reactive gliosis).

Topographic genotyping integrates anatomic pathology and molecular analyses. Briefly, it involves performing microscopic examination of a specimen, identifying areas of interest on the pathology slide, and microdissecting (manually excising) them under the microscope. The minute tissue samples, enriched in tumor cells, can be subjected to molecular analyses with specifically developed protocols.

It has been claimed that analyzing microdissected tissue areas using specific genetic marker panels can aid pathologic diagnosis, individualize prognosis and guide treatment decisions. Herein we perform a systematic review of the published literature on loss-of-heterozygosity based topographic genotyping with PathfinderTG®, a patented technology for topographic genotyping offered by the private company RedPath Integrated Pathology Inc. (www.redpathip.com).

Methods

The following key questions were asked:

1. What is the published evidence on the analytic test performance (analytic sensitivity and specificity) of loss-of-heterozygosity based topographic genotyping with PathfinderTG® compared to a gold standard test (Fryback Level 1)?
2. What is the published evidence on the diagnostic ability and clinical validity of loss-of-heterozygosity based topographic genotyping with PathfinderTG® (Fryback Levels 2, 3, 4 and 5)?
3. What is the direct evidence comparing loss-of-heterozygosity based topographic genotyping with PathfinderTG® with conventional pathology without this process for clinical outcomes?
4. Does the study indicate whether informed consent was given, whether Institutional Review Board (IRB) approval was obtained, and whether institutional guidelines for human subject protection were considered in the design or implementation of the study,
5. For published studies of loss-of-heterozygosity based topographic genotyping with PathfinderTG® does the study indicate how the study population relates to the Medicare beneficiary population (for example, by providing an age or age-group breakdown or profile of the study population)?

Eligible were only studies evaluating the patented technology, and more specifically, those using loss of heterozygosity (LOH) analysis. In addition, eligible studies on analytic validity and on diagnostic and predictive ability were required to have a suitable reference standard. We excluded studies with less than 10 patients, studies that did not use LOH analyses on microdissected samples, and studies that described

molecular differences of different tumor subgroups without quantifying the diagnostic or prognostic ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® (as an overall diagnostic process) against a suitable reference standard.

Results

Key Question 1

We did not identify any studies on the analytic validity of loss-of-heterozygosity based topographic genotyping with PathfinderTG®. The laboratory that performs these analyses is CLIA (Clinical Laboratory Improvement Amendments) and New York State certified and College of American pathologists accredited to perform high complexity analyses. The data from these certifications and accreditations are generally not publicly available.

Key Question 2

We identified 15 eligible publications. These pertained to lung cancer (n=4), pancreatic and biliary tree tumors (n=4), hepatocellular carcinoma (n=4), gliomas, thyroid tumors, lacrimal gland tumors and mucinous tumors of the appendix (n=1 for each). Sample sizes ranged from 11 to 103.

Microdissection and molecular analysis protocols were described in detail in all studies. However, details on patient (sample) selection, patient characteristics, treatments received, clinical endpoint definitions, justification of sample size, selection of classification thresholds (for molecular analysis aggregate scores such as fractional allelic loss (FAL)), selection among various statistical models, and other important parameters of study design and reporting were provided inconsistently.

All studies were retrospective in design and used available archival tissue blocks. In a single study, molecular profiles of gliomas and reactive gliosis were determined retrospectively, and then they were used *prospectively* on 16 diagnostically challenging cases of reactive gliosis versus glial tumors.

Three publications pertained to diagnostic accuracy: Two examined the ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® to diagnose malignancy from pancreatico-biliary cytology specimens, and one its ability to diagnose reactive gliosis versus glioma. The reference standard was pathological confirmation of surgical specimens or long-term clinical followup, as applicable.

The remaining 10 publications evaluated the association of loss-of-heterozygosity based topographic genotyping with PathfinderTG® aggregate scores and survival, recurrence-free survival, and tumor recurrence. Retrospectively collected clinical data were used to ascertain outcomes. Overall, studies used different and arbitrary cutoffs in the FAL to classify studies, did not adjust for treatment or other predictors of outcome and did not provide multivariate analyses. In seven out of 10 publications FAL was identified as a statistically significant predictor of a clinical outcome in at least one analysis or subgroup (and for the presented cutoffs).

Key Question 3

We did not identify any eligible studies.

Key Question 4

All studies except for two specifically mentioned that IRB approval was obtained. However, during the peer review period, the company clarified that all studies were conducted following institutional mandates.

Key Question 5

No study explicitly stated its applicability to the Medicare beneficiary population. Five studies did not provide any information on age distributions. In the remaining eight studies, mean ages ranged from 48 to 70 years (above 60 years in six studies).

Overview

Most studies of loss-of-heterozygosity based topographic genotyping with PathfinderTG® were excluded because they only described the molecular profile of different tumors, without assessing the ability of the method to help improve making diagnosis or prognosis. There were no studies that directly inform on the effect of using loss-of-heterozygosity based topographic genotyping with PathfinderTG® on patient-relevant clinical outcomes. Eligible studies on the diagnostic and prognostic ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® were small in sample size, had overt methodological limitations, and did not clearly report important characteristics of their study design. Most studies clearly reported receiving IRB approval. Evaluating the applicability of studies to the Medicare beneficiary population was hindered by the lack of details on patient characteristics. Introduction

Microscopic analysis of tissue samples is central to the diagnosis and management of patients with malignancy. There are several instances where the morphologic analysis of a tissue specimen is inconclusive and may not be able to inform management decisions. For example, two morphologically similar tumors in the same patient may or may not have arisen from the same tumor (i.e., one tumor may be a *de novo* independent tumor from the already diagnosed tumor),[1] or in some instances where it is difficult to distinguish malignant from nonmalignant tissue (e.g., some glial neoplasms from reactive gliosis[2]).

Molecular testing in anatomic pathology has emerged as a means to address many difficulties in the diagnosis of disease. Some researchers have also advocated that it can provide better prognosis and facilitate treatment guidance.

One of the technical difficulties of molecular testing in anatomic pathology stems from the fact that tumors are heterogeneous, i.e., tumor tissues from a pathological specimen contain various subpopulations of cells. Methods such as flow cytometry can separate cellular subpopulations based on their phenotypes, but they do not allow for concurrent microscopy analyses, and they typically require large quantities of fresh tissues.[3] An alternative approach is to perform microscopic examination of a specimen, identify areas of interest on the pathology slide, and to microdissect (manually excise) them under the microscope. This will allow one to obtain minute tissue samples that are enriched in tumor cells and can be used for molecular analyses with specifically developed protocols.

It has been claimed that analyzing microdissected tissue areas using specific genetic marker panels –*topographic genotyping*– can aid pathologic diagnosis, individualize prognosis and guide treatment decisions. Herein we perform a systematic

review of the published literature on loss-of-heterozygosity based topographic genotyping with PathfinderTG®, a patented technology for topographic genotyping offered by the private company RedPath Integrated Pathology Inc. (www.redpathip.com).

The aim of the systematic review is to describe the published evidence on the analytic validity of loss-of-heterozygosity based topographic genotyping with PathfinderTG®, as well as the published evidence on its clinical validity and utility.

Methods

This Technology Assessment focuses specifically on the patented RedPath PathfinderTG® technology, namely loss-of-heterozygosity based topographic genotyping with. In discussions with the cofounder and Chief Scientific Officer of RedPath, Dr. Sydney Finkelstein, it was clarified that only papers (co)authored by Dr. Finkelstein have used the patented technology (see below for a description). Therefore, only published English-language studies in which Dr Sydney Finkelstein is among the authors were eligible for the Technology Assessment.

The Technology Assessment follows the Fryback and Thornbury framework for assessing studies that evaluate diagnostic tests.[4] The framework distinguishes six levels for the evaluation of diagnostic technologies.

1. Technical feasibility and optimization
2. Diagnosis (i.e., diagnostic accuracy, or prognostic accuracy)
3. Impact on diagnostic thinking (e.g., ordering new tests)
4. Impact on therapeutic choices (e.g., choosing a treatment over another)
5. Impact on patient outcomes (e.g., in a clinical trial)
6. Societal impact (e.g., a cost-effectiveness analysis)

The framework was developed for imaging tests, and does not necessarily apply equally well to the evaluation of PathfinderTG®. However, the rationale of the framework is generic, and we were able to use it by applying lenient criteria to assign identified studies to levels in the framework.

Task order

The Coverage and Analysis Group at the Centers for Medicare and Medicaid Services (CMS) requested an assessment on the use of PathfinderTG® (a patented technology) for disease diagnosis and prediction of clinical outcomes from The Technology Assessment Program (TAP) at the Agency for Healthcare Research and Quality (AHRQ). AHRQ assigned this report to the Tufts Evidence-based Practice Center: (Contract No. HHSA 290 2007 10055 I). After discussions with AHRQ and CMS and after exploration of the literature, five key questions were formulated (see next paragraph).

Key questions

Key question 1

What is the published evidence on the analytic test performance (analytic sensitivity and specificity) of PathfinderTG® compared to a gold standard test (Fryback Level 1)?

This key question pertains both to the microdissection and to the molecular analytic stage (specifically LOH analysis) of loss-of-heterozygosity based topographic genotyping with PathfinderTG®. (See definition of LOH in later part of this section.)

More specifically, there are 3 subquestions:

Subquestion 1.1: What is the test performance (sensitivity and specificity) of loss-of-heterozygosity based topographic genotyping with PathfinderTG® compared with "gold standard" tests for the detection or quantitation of genetic characteristics of a specimen?

a. Document potential bias or potential technical limitations in loss-of-heterozygosity based topographic genotyping with PathfinderTG® diagnostic testing methods.
b. Describe the "gold standard" used to determine that one or more genetic characteristic(s) is (are) present or not. Are there published reviews of the used "gold standard" test method, its limitations if any? Describe the population(s) in which the gold standard can be reliably used to detect or quantify a genetic characteristic, disease, or condition.

Subquestion 1.2: Are there published studies on the reliability (repeatability and reproducibility) of the assessment of loss-of-heterozygosity based topographic genotyping with PathfinderTG® results?

Subquestion 1.3: If an algorithm, decision tree, calculation, or other interpretation procedure is used in assessing loss-of-heterozygosity based topographic genotyping with PathfinderTG® results:

c. Are there published studies about how the algorithm, decision tree, or other interpretation procedure was developed?
d. Are there published studies about how the algorithm, decision tree, or other interpretation procedure was validated in a previously untested population?
e. Are there published studies about how the algorithm, decision tree, or other interpretation procedure compared to reference standard diagnostic studies used for the same purpose (e.g., panel of tumor markers for prognosis)?

Key question 2

What is the published evidence on the diagnostic ability and clinical validity of loss-of-heterozygosity based topographic genotyping with PathfinderTG® (Fryback Levels 2, 3, 4 and 5)?

Diagnostic ability (Fryback Level 2) pertains to the sensitivity and specificity of loss-of-heterozygosity based topographic genotyping with PathfinderTG® to diagnose a disease as ascertained by a reference standard. Clinical validity is the ability to affect patient outcomes. Here it is considered as Levels 3 to 5 in the Fryback framework, that is, the ability to impact on diagnostic thinking (Level 3, e.g., number of times a diagnosis changed after the examined diagnostic process was implemented), impact on therapeutic decision (Level 4, e.g., number of times patient management changed after loss-of-heterozygosity based topographic genotyping with PathfinderTG® was implemented), and the ability to affect patient outcomes (Level 5, e.g., changes in patient-relevant clinical outcomes when loss-of-heterozygosity based topographic genotyping with PathfinderTG® testing was implemented).

Key question 3
What is the direct evidence comparing loss-of-heterozygosity based topographic genotyping with PathfinderTG® with conventional pathology without PathfinderTG® for clinical outcomes?

This key question focuses on *comparative* studies (of using versus not using loss-of-heterozygosity based topographic genotyping with PathfinderTG®) for patient-relevant clinical outcomes. Such studies fall into Level 5 in the Fryback classification.

Key question 4
For published studies of loss-of-heterozygosity based topographic genotyping with PathfinderTG® involving human subjects, does the study indicate whether:

a. *Informed consent was given by subjects participating in the evaluation of loss-of-heterozygosity based topographic genotyping with PathfinderTG® diagnostic techniques?*
b. *IRB approval was needed for the study?*
c. *Institutional guidelines for human subject protection were considered in the design or implementation of the study?*

In discussions with CMS and AHRQ, it was decided to examine this key question for studies eligible for key questions 1 to 3.

Key question 5:
For published studies of loss-of-heterozygosity based topographic genotyping with PathfinderTG®, does the study indicate how the study population relates to the Medicare beneficiary population (for example, by providing an age or age-group breakdown or profile of the study population)?

In discussions with CMS and AHRQ, it was decided to examine this key question for studies eligible for key questions 1 to 3.

Eligibility criteria

General criteria

Inclusion criteria

Eligible were only studies fulfilling the following criteria:

1. Evaluating the patented loss-of-heterozygosity based topographic genotyping with PathfinderTG® technology.
   As mentioned above, in communications with the company and its Chief Scientific Officer, it was clarified that Dr. Sydney Finkelstein would be an author or coauthor of all such papers.
2. Using loss of heterozygosity (LOH) analysis on microdissected samples.
   For example, we excluded studies that performed LOH analyses on DNA extracted from pancreatic cyst fluid by means of column separation (without using microdissection to isolate cells from the cystic fluid).
   The performance of loss-of-heterozygosity based topographic genotyping with

PathfinderTG® as a diagnostic or prognostic tool depends also on the typed genetic marker panels. Since the focus of this Technology Assessment is on LOH-based analyses, we did not consider studies that focused only on the detection of mutations of *K-ras-2* or *P53* (unless they also included a panel of microsatellite markers and provided a single "aggregate" score/risk/disposition per patient – see below). Furthermore, the majority of papers examining the prognostic and diagnostic ability of mutations in the *P53* and *K-ras-2* genes in cancer are not included in our Technology Assessment (because Dr. Finkelstein is not among the authors – an operational criterion to identify studies that use the patented technology).

3. At least 10 analyzed patients.

Exclusion criteria

We excluded studies that described molecular differences of different tumor subgroups without quantifying the diagnostic or prognostic ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® (as an overall test) against a suitable reference standard.

Specific criteria for Key Questions 1 and 2

In addition, after discussions with AHRQ and CMS, the following eligibility criteria were set for Key Questions 1 and 2:

For Key Question 1:

1. Experimental studies that can quantify the analytic sensitivity and the analytic specificity of loss-of-heterozygosity based topographic genotyping with PathfinderTG® to detect LOH in samples in which known LOH state was based on a reference method.
2. The design of the study should be such that it controls for the effect of potential confounders (e.g., within-tissue heterogeneity).
3. The reference method would preferably be Copy Number Variation (CNV) analysis,[5] or any other reference method chosen by the authors.

Eligible for Key Question 2 were single arm noncomparative studies (N>=10) or comparative studies (comparing using loss-of-heterozygosity based topographic genotyping with PathfinderTG® versus not) for diagnosis, prognosis or treatment guidance.

We excluded studies that only described the molecular profile of tumors without evaluating the accuracy of loss-of-heterozygosity based topographic genotyping with PathfinderTG® diagnosis or risk stratification against a reference standard. For example, studies on diagnostically challenging tumors are excluded, unless they have data from long enough clinical followup. Similarly, studies on inconclusive cytology specimens are excluded, unless they are verified against surgical biopsy, adequate clinical followup or other extensive workup.

Finally, we excluded studies that did not use any LOH analyses; and studies that did not use an aggregate score for all genetic markers, but instead described each molecular marker separately. An aggregate score could be anything that integrates information from the whole loss-of-heterozygosity based topographic genotyping with PathfinderTG® diagnostic process, either by simply using the proportion of markers

exhibiting LOH, or using more sophisticated approaches (e.g., artificial neural networks, or other classifiers).

Terminology and definitions

Microdissection

Microdissection (**Figure 1**) is the process of extracting a microscopic tissue area from a tissue sample, typically from a pathology slide. By extension, it is the selection of a subset of cells from a brush cytology specimen. Microdissection can be manual, or laser assisted.

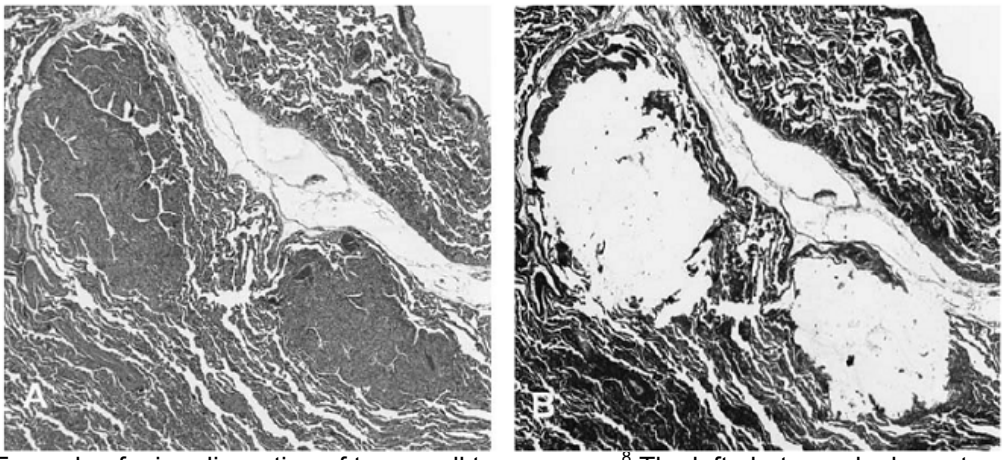
First, the specialist (pathologist) selects tissue areas with high purity of tumor cells on a tissue slide. Manual microdissection is based on the fact that dry and wet tissue on a pathology slide separate cleanly: The area of interest is first dampened slightly with a small amount of buffer solution and then scraped off with the edge of a scalpel under stereomicroscopic[a] visualization.[3]

In laser microdissection, the specialist uses a laser beam to separate individual cells from the adjacent tissues. Different systems for laser microdissection exist. One system uses lasers to attach individual cells on a special thermoplastic membrane overlaid on the tissue slide. When the membrane is lifted from the tissue, the selected cells remain attached to it and are effectively "microdissected". Other systems use the laser beam to "cut" the area of interest from the surrounding tissue, so that it will fall off by gravity. Laser microdissection is reported as accurate enough to extract individual cells from a tissue slide.[3]

Finally, there are other microdissection methods that have been described,[3] including methods that use craft glue,[6] or tissue glue[7] that adheres on the area of interest, and detaches it (the area of interest) from the slide when it is "peeled" off.

[a] It is possible to use an ordinary microscope also. However, the image in the ordinary microscope is inverted and for some magnifications the distance between the microscope lens and the glass slide is too small to allow for easy microdissection.[3]

Figure 1. Manual microdissection of a small tumor area from a pathology slide



Example of microdissection of two small tumor areas.[8] The left photograph shows two minute pulmonary meningothelial-like nodules (small nodules in the lung) stained with hematoxylin and eosin (a "scout" section[3] to guide the microdissection of the unstained tissue on the right); the right photograph is an unstained adjacent tissue slide after the manual microdissection of the areas corresponding to the two nodules.
(Reproduced with permission from Ionescu D et al. Am J Surg Pathol. 2004;28:207-14, Figure 1)

Topographic genotyping

Topographic genotyping integrates microscopic analysis and microdissection with genomic mutational profiling of the microdissected areas.

The microdissected tissue areas are enriched in tumor cells. The DNA extracted from these samples is therefore not greatly contaminated by DNA from nonmalignant cells (e.g., from supporting stromal cells). Genetic (molecular) analyses of the microdissected samples can provide a molecular profile of malignant cells, i.e., they inform on the presence or absence of selected genetic variations in malignant cells.

This combination of microdissection and genetic analyses on the extracted tumor areas has been used by several researchers and is not a unique characteristic of the PathfinderTG® diagnostic process.

PathfinderTG®

PathfinderTG® is a patented technology for topographic genotyping and other molecular analyses. The patented technology pertains to the ability for enhanced amplification of genetic material from minute tissue samples, even from archival blocks of fixative-treated samples or unstained pathology slides.

Microsatellite markers

Microsatellites are a type of common variation in the genetic material. Other types of genetic variations include single nucleotide polymorphisms (SNPs) and insertions/deletions (Ins/Del).

Microsatellites are short repeated DNA sequences that are found in various loci throughout the human genome. Examples of such repeats are TATA…TA, *n* times [commonly denoted as (TA)*n*]. Different "alleles" of a specific microsatellite marker essentially differ in the number of repeats, *n*. Microsatellite markers are highly

polymorphic, because *n* can range between 4 and 40 for many markers. Especially for this reason (their high polymorphism), they are often used as markers in genetic analyses.

In contrast, SNPs are common single-base changes in specific locations in the genome. A SNP is a biallelic marker (the two alleles are the bases that change).

Loss of heterozygosity (LOH)

Cellular DNA is constantly damaged and repaired. Generally speaking, cancers can arise when excessive DNA alterations accumulate beyond the restoration capacity of repair mechanisms. Loss of heterozygosity (LOH) can be a telltale sign that DNA repair mechanisms (*homologous recombination* in particular –see below) have acted in specific regions in the DNA.

LOH is a frequent genetic alteration in many neoplasms.[9] It is theorized that LOH alterations that co-locate in specific genomic regions (e.g., near genes implicated in the pathogenesis of malignancy) may have prognostic significance (they imply DNA repair activity near these genes).[9] In addition, observing increased rates of LOH is considered an indication of inactivation of tumor suppressor genes. Most often, LOH is evaluated for microsatellite markers.

Homologous recombination and LOH

In our somatic cells we have two chromosomal homologues (two "copies" of each DNA macromolecule). When either one of them is damaged, the cell repairs it using several mechanisms. *Homologous recombination* (or *general recombination*) is one DNA repair mechanism that corrects the damaged chromosomal homologue using the other as a template. Homologous recombination corrects many DNA repair problems, but can introduce changes to the DNA. Assuming that the two chromosomal homologues differ at (or very near) a DNA site that has been damaged (i.e., the person is *heterozygous* at that position), homologous recombination will make both chromosomal analogues homozygous (identical) at the repaired site and near it, because it uses one DNA strand as the template to correct the other. Effectively, there is a *loss of heterozygosity*. It is well appreciated that excessive homologous recombination can lead to malignancies because of increased loss of heterozygosity.[10]

Fractional allelic loss (FAL)

FAL is the proportion of informative (heterozygous) microsatellites that exhibit LOH. It is a simple aggregate score. Other methods of obtaining a diagnostic or prognostic score (information) of a panel of markers exist (e.g., with neural networks, logistic regression, support vector machines or other techniques).

Results

Eligible studies

We examined 155 papers in which Dr Finkelstein was among the co-authors in full text. According to the pre-specified criteria, 15 reports were eligible for this Technology Assessment.

Key Question 1: What is the published evidence on the analytic test performance (analytic sensitivity and specificity) of loss-of-heterozygosity based topographic genotyping with PathfinderTG® compared to a gold standard test (Fryback Level 1)?

None of the included studies evaluated the analytic validity of microdissection or LOH analyses in the PathfinderTG® framework compared to a reference standard.

Subquestion 1.1

We did not find data informing on the sensitivity and specificity of the microdissection or the LOH-based molecular analysis component of PathfinderTG® among the eligible papers.

The laboratory that performs these analyses is CLIA (Clinical Laboratory Improvement Amendments) and New York State certified and College of American pathologists accredited to perform high complexity analyses. The data from these certifications and accreditations are generally not publicly available.

Subquestions 1.1(a) and (b)

The authors made the following comments and clarifications:

- The authors of the papers reviewed noted that manual microdissection was successful in obtaining tissue slide areas with sufficient purity in tumor cells to allow for successful topographic genotyping.
- The authors of the paper reviewed reported following protocols developed to avoid the phenomenon of *allelic dropout* (ADO) during LOH analyses. ADO can occur during PCR amplification, when nucleic acid amplification preferentially favors one allelic DNA template over the other, because of limiting quantities of starting DNA in the microdissected samples.[11,12] The phenomenon was originally described in the context of single-cell PCR, but has been described in situations where genotyping is performed on biopsy samples of fixed tissue specimens.[13] The authors reported avoiding ADO by microdissecting equally-sized tumor and normal tissue areas for LOH analyses.

Subquestion 1.2

There were no eligible studies on the test-retest repeatability and reproducibility of results (either within batch or between runs repeatability, or on test reproducibility across laboratories).

Subquestion 1.3

Regarding the development of algorithms and interpretation procedures in the assessment of PathfinderTG® results, please refer to Key Question 2.

Key Question 2: What is the published evidence on the diagnostic ability and clinical validity of loss-of-heterozygosity based topographic genotyping with PathfinderTG® (Fryback Levels 2, 3, 4 and 5)?

We identified 15 eligible publications. These pertained to lung cancer (n=4),[14-17] pancreatic and biliary tree tumors (n=4),[18-21] hepatocellular carcinoma (n=3),[1,22,23] gliomas,[2] thyroid tumors,[24] lacrimal gland tumors[25] and mucinous tumors of the appendix[26] (n=1 for each).

Characteristics of study design

All studies were retrospective in design and used convenience sampling, namely archival tissue blocks that were available. It is unknown how many patients with similar clinical status for whom samples were not available, and whether they had systematically different characteristics from those analyzed (sampling bias). The authors did not report efforts to correct for sampling bias in any study.

In a single study, molecular profiles of gliomas and reactive gliosis were determined retrospectively, and then they were used *prospectively* on 16 diagnostically challenging cases of reactive gliosis versus glial tumors.[2]

Sample sizes ranged from 11 to 103 patients, with only three publications reporting on more than 50 patients.[1,22,23] It is possible that these three publications analyzed the same patient population (patients with hepatocellular carcinomas) with different methods.

Characteristics of included patients

All study populations were convenience samples. With one exception,[2] all studies included only archival tissue samples that were available for analyses.

As shown in **Table 1a, 1b and 1c**, most studies did not describe patient characteristics in detail. For example, basic information on age (mean or median age and standard deviation or range) was not given in seven of 15 studies.[1,2,19,21-23,25] Five studies reported no information on patients' treatments.

Table 1a. Characteristics of patients with gastrointestinal tumors in the included studies.

Author Year [PMID]	N	Description	Histology and stage	Age	Treatments
Cong 2001 [11260864]	22	Paraffin blocks from surgical resections of CC (1989-1999); no prior chemo- or radiotherapy	CC T: 1-3 N: 0-2 M: nd	Mean age 63 y; 11/22 (50%) ≥ age 65	Surgery[b]
Finkelstein 2003 [12668980]	103	Paraffin blocks from primary HCC (1988-1996); hepatic transplant recipients with > 5 y follow-up	HCC	Not stated	Hepatic transplant
Marsh 2003 [12827550]	103	Paraffin blocks from primary HCC[c] (1988-1996); hepatic transplant recipients with > 5 y follow-up	HCC	Not stated	Hepatic transplant
Khalid 2004 [15542529]	26	Papanicolaou-stained smears of pancreatico-biliary brush cytology[d] of patients suspected of having malignancy (inferred)	PDC (n=6) CC (n=11) Inflammatory (n=9)	Not stated	NA
Khalid 2006 [17029619]	21	Papanicolaou-stained smears of pancreatico-biliary cytology obtained by EUS-FNA‡ of patients suspected of having malignancy (inferred); either positive or inconclusive cytology cases only	PDC (n=15) ACC (n=1) CC (n=1) AIP/PIN (n=1) CP (n=2) AIP (n=3)	Mean age 66 y; 12/2 (57%) ≥ age 65	NA
Schwartz 2008 [18602719]	70	Archival tissue from consecutive patients after liver transplantation for HCC (35 fulfilling Milan criteria, 35 beyond)[e]	HCC	Mean age 55.3, (SD = 10.4)	Hepatic transplant
Fasanella 2009 [19152901]	29[f]	Patients with pancreatic endocrine tumors (PET) with molecular analyses. Excluded were patients with other malignancies or stable disease with <1 year follow up.	Pancreatic endocrine tumors (PET)	57 (range 31-80)	Surgery in 23/29

ACC: acinar cell carcinoma; AD: adenocarcinoma; AIP: autoimmune pancreatitis; BAC: broncholveolar carcinoma; DPAM: Disseminated peritoneal adenomucosis; CC: cholangiocarcinoma; CP: chronic pancreatitis; EUS: endoscopic ultrasound; FNA: fine needle aspirate; HCC: hepatocellular carcinoma; IACC: intraarterial cytoreductive chemotherapy

[b] No details reported regarding surgical treatment and adjuvant chemo- or radiotherapy.
[c] Primary diagnosis: hepatitis C (n=30), hepatitis B (n=24), cryptogenic cirrhosis (n=16), alcoholic hepatitis (n=13), hemochromatosis (n=4), alpha-1 antitrypsin deficiency (n=2), primary biliary cirrhosis (n=2), autoimmune hepatitis (n=1),primary sclerosing cholangitis (n=1)
[d] DNAs from paraffin blocks from surgical resections were also evaluated.
[e] Based on histology only.
[f] 27 and 25 reported in tables and figures, depending on outcome described

Table 1b. Characteristics of patients with lung tumors in the included studies.

Author Year [PMID]	N	Description	Histology and stage	Age	Treatments
Sasatomi 2002 [11980668]	48	Paraffin blocks from pts with NSCLC[g] (1994-98); ≥2 y followup	Stage II NSCLC	Mean age: 66.4 y; 34/48 (71%) ≥ age 65	Not stated
Sasatomi 2004 [15371943]	34	14 cases of BAC & 20 cases of stage I AD	nonmucinous or mucinous BAC; G1-3 AD	BAC: mean age: 64 y; 7/14 (50%) ≥ age 65 Stage I AD: mean age: 67; 10/20 (50%) ≥ age 65	Surgery
Dacic 2005 [15958854]	20	Paraffin blocks from pts with adenocarcinoma of the lung	Invasive (pathologic stage T4)	mean age: 70 y; 13/20 (65%) ≥ age 65	Surgery [excluded those with preoperative chemotherapy or irradiation]
Fernando 2004 [14752417]	40	stage II NSCLC with standard pulmonary resection; adenocarcinoma or squamous carcinoma only; excluded pts with wedge or segmental resection; excluded T3N0 tumors	stage II NSCLC with affected N1 lymph nodes	median age: 68; range 42-85	Lobectomy or pneumonectomy

ACC: acinar cell carcinoma; AD: adenocarcinoma; AIP: autoimmune pancreatitis; BAC: broncholveolar carcinoma; DPAM: Disseminated peritoneal adenomucosis; CC: cholangiocarcinoma; CP: chronic pancreatitis; EUS: endoscopic ultrasound; FNA: fine needle aspirate; HCC: hepatocellular carcinoma; IACC: intraarterial cytoreductive chemotherapy

[g] non-small cell lung carcinoma

Table 1c. Characteristics of patients with other tumors in the included studies.

Author Year [PMID]	N	Description	Histology and stage	Age	Treatments
Sheikh 2004 [14707871]	11	Archival cases with paraffin blocks available	Medullary thyroid carcinoma (sporadic); Stage II, III, IV	Mean age 48 [range: 17-72]; 5/11 men	Not stated
Maheshwari 2006 [17009159]	23	Archival cases with paraffin blocks available	Pseudomyxoma peritonei; DPAM (benign), PMCA (aggressive), intermediate	Mean age 54 [range: 27-90]; 17/23 men	Debulking surgery +/- intraperitoneal chemotherapy (n=22)
Tse 2006 [16386976][h]	16	Archival cases with paraffin blocks available	Lacrimal gland adenoid cystic carcinoma	Not stated	Chemotherapy (IACC) and orbital exenteration and chemoradiotherapy (n=9) Conventional therapies (n=7)[i]
Finkelstein 2004 [15151207]	16[j]	Prospective cases with challenging diagnosis between reactive gliosis and glioma	[Patients in whom pathologic diagnosis between reactive gliosis and glioma was difficult]	Not stated	Not stated

ACC: acinar cell carcinoma; AD: adenocarcinoma; AIP: autoimmune pancreatitis; BAC: broncholveolar carcinoma; DPAM: Disseminated peritoneal adenomucosis; CC: cholangiocarcinoma; CP: chronic pancreatitis; EUS: endoscopic ultrasound; FNA: fine needle aspirate; HCC: hepatocellular carcinoma; IACC: intraarterial cytoreductive chemotherapy

[h] Described as retrospective case series, comparative.

[i] Only 7 of 16 patients treated with conventional therapy between 1967 and 1994 had available tissue blocks for this study

[j] 85 samples in total. Molecular profiling was applied *prospectively* to 16 diagnostically challenging cases of reactive gliosis versus glioma; the study included *retrospective analysis of* 15 cases of clear reactive gliosis and 54 cases of various gliomas to check molecular profiles in these 2 entities, and then applied molecular analyses in the 16 challenging cases.

Topographic genotyping

Manual microdissection was used in all studies. The examined genetic markers were microsatellites near genes implicated in carcinogenesis. **Table 2a, 2b, and 2c** list the examined microsatellites and genes in their proximity.

LOH was defined similarly in all studies. Typically, the operational definition of positive LOH was that the corresponding allelic peaks from tumor and normal tissue areas differed at least two-fold. FAL was the aggregate score in all studies. FAL is the proportion of informative microsatellites that have LOH. Some studies also examined mutations in *K-ras* (no details on which mutations). The presence of such mutations was taken into account together with the LOH markers in the construction of the aggregate score (which was termed "fractional mutation rate").

No studies examined other ways to utilize the genetic information (e.g., using all markers in a multivariate model as individual predictors, or constructing a different score). However, FAL is a simple aggregate score, and the studies were of limited sample size to allow for meaningful exploration of alternative analyses.

FAL cutoffs to classify tumors differed across studies (e.g., FAL>0 or above and below the median) and appeared to be selected post-hoc. None was validated in an independent sample. A single study[21] reported using a FAL cutoff determined in previous analyses.[19] However, it is likely that patients included in these studies overlap extensively.

Only three studies clearly reported any validation of the reliability of LOH analyses (concordance between different readers or in repeat testing). Four publications clearly stated that the pathologist was blinded to clinical information[1,20,23,25] (two[1,23] likely referred to the same patients).

Table 2a. Characteristics of genetic analyses in studies of gastrointestinal cancers.

Author Year [PMID]	Marker panel	Nearby genes	Definitions of marker positivity	Aggregate score; cutoff	Validation or reliability	Blinding of assessor
Cong 2001 [11260864]	Not stated	*APC* *DCC* *OGG1* *p53*	• Positive LOH: >80% reduction of radiographic signal intensity of a polymorphic allele compared with normal	• FAL	Not stated	Not stated
Finkelstein 2003 [12668980]	D1S407 MYCL D3S1539 D3S2303 D5S592 D5S615 MCC DS71530 D8S373 D9S251 D9S254 D10S520 D10S1173 TP53 D17S974 D17S1289 D17S1163 D18S814	Not stated	• Positive LOH: The ratio of allelic peak heights > 2.00 or < 0.50	• FAL	Not stated	Blinded to clinical and pathologic al data
Marsh 2003 [12827550]	D1S407 MYCL D3S1539 D3S2303 D5S592 D5S615 MCC DS71530 D8S373 D9S251 D9S254 D10S520 D10S1173 TP53 D17S974 D17S1289 D17S1163 D18S814	See footnote[k]	• Positive LOH: The ratio of allelic peak heights > 2.00 or < 0.50	• FAL	Validation was performed in 22 patients (concordanc e > 50%)	Blinded to clinical (?and pathologic al) data
Khalid 2004 [15542529]	D1S407 MYCL	*CMM/RIZ*	• Positive LOH: The ratio of allelic peak heights > 2.00 or < 0.50	• FAL (modified to include *K-ras* mutations)	Not stated	Not stated
	D3S1539 D3S2303	*VHL*				
	D5S592 D5S615	*APC*				
	D9S251 D9S254	*CDKN2A/p16*				
	D10S520 D10S1173	*PTEN*				
	D17S974 D17S1289	*p53*				
	"Point	*K-ras**				

	mutations"					
Khalid 2006 [17029619]	D1S407 MYCL	*CMM/RIZ*	• LOH: The ratio of allelic peak heights falling outside of two standard deviations beyond the mean for allele pairing	• FAL (modified to include *K-ras* mutations)	Not stated	Blinded to patient identity and diagnosis
	D3S1539 D3S2303	*VHL*				
	D5S592 D5S615	*APC*				
	D9S251 D9S254	*p16*				
	D9S252	*PTCH*				
	D10S520 D10S1173	*PTEN*				
	D17S974 D17S1289	*p53*				
	D17S1161	*Her2/neu*				
	D21S1244	*ETS2*				
	D22S532	*NF2*				
	"Point mutations"	*K-ras*				
Schwartz 2008 [18602719]	MYCL 5NT	*L-MYC*	• Positive LOH: When the ratio of the individual allele peaks fell outside the range of 0.66 to 1.50.	• FAL • Cutoff was 0.27, based on the ROC curve that discriminates recurrence from non-recurrence in the studied population.	Replicate analysis performed in every case. Concordance of 85-100% and SD from 0.06 to 0.20.	Not stated
	D1S407	*[NONE]*				
	D3S2303	*OGG1*				
	D3S1539	*OGG1*				
	MCC E10	*MCC*				
	D5S592	*APC*				
	D5S615	*APC*				
	D7S1530	*MET*				
	D8S373	*C-MYC*				
	D9S251	*CDKN2A*				
	D9S254	*CDKN2A*				
	D10S520	*PTEN*				
	D10S1173	*PTEN*				
	D17S1289	*TP53*				
	D17S974	*TP53*				
	TP53 L1	*TP53*				
	D17S1163	*[NONE]*				
	D18S814	*DCC*				
Fasanella 2009 [19152901]	[broad panel of markers located on chromosomal arms 1p, 3p, 5q, 9p, 10q, 11q, 17p, 17q, 21q and 22q]	*[not stated]*	• Positive LOH: When the ratio of the individual allele peaks fell outside 2 SDs beyond the mean.	• FAL • Cutoff was 0.2, based on previous analyses (?)[l]	Not stated	Not stated[m]

FAL: fractional allelic loss; SD: standard deviation

Table 2b. Characteristics of genetic analyses in studies of lung cancers.

Author Year [PMID]	Marker panel	Nearby genes	Definitions of marker positivity	Aggregate score; cutoff	Validation or reliability	Blinding of assessor
Sasatomi 2002 [11980668]	D1S407	*CMM*	• Positive LOH: ratio between informative allelic band heights of <0.5 or >2.0; LOH of a chromosome region is positive if any of the microdissected sample showed LOH	• FAL • If any had FAL (>0.0)	Concordance ratio of positive LOH between 2 independent observers was 100%	Not stated
	MYCL1[n]	*MYCL1*				
	D3S1539	*VHL*				
	D3S2303					
	D5S592	*APC*				
	D5S615	*MCC*				
	MCC[d]					
	D7S1530	*MET*				
	D8S373	*MYC*				
	D9S254	*CDKN2A*				
	D9S251					
	D10S520	*PTEN*				
	D10S1173	*MX11*				
	D17S1163	*TP53*				
	TP53[d]					
	D18S814	*DCC*				
Sasatomi 2004 [15371943]	D1S407	*CMM*	• positive LOH: ratio between informative allelic band heights of <0.5 or >2.0; LOH of a chromosome region is positive if any of the microdissected sample showed LOH	• FAL	Not stated	Not stated
	MYCL1[d]	*MYCL1*				
	D3S1539	*VHL*				
	D3S2303					
	D5S615	*APC*				
	MCC[d]	*MCC*				
	D7S1530	*MET*				
	D8S373	*MYC*				
	D9S251	*CDKN2A*				
	D9S254					
	D10S1173	*MX11*				
	D10S520	*PTEN*				
	D17S974	*TP53*				
	D17S1289					
	D18S814	*DCC*				
Dacic 2005 [15958854]	D1S407	*CMM*	• positive LOH: ratio for the specific microsatellite marker was <0.6 or >1.5	• FAL • ≤0.40 vs. >0.40	Not stated	Not stated
	D1S1193					
	MYCL1	*MYCL1*				
	D3S1539	*VHL*				
	D3S2303					
	D5S592	*APC*				
	D5S615					
	D9S251	*CDKN2A*				
	D9S254					
	D9S252	*not applicable*				
	D10S1173	*MX11*				
	D10S520	*PTEN*				
	D17S974	*TP53*				
	D17S1289					
	D22S532					
Fernando 2004 [14752417]	D1S407	*L-myc*	• LOH: diminished band activity ≥50% of the other corresponding bands for chromosome loci that were informative	• FAL	Not stated	Not stated
	MYCL1					
	D3S1539	*OGG1*				
	D3S2303					
	D5S592	*APC*				
	D5S615					
	MCC.E10					
	D7S1530	*c-MET*				

Table 2c. Characteristics of genetic analyses in studies of other tumors.

Author Year [PMID]	Marker panel	Nearby genes	Definitions of marker positivity	Aggregate score; cutoff	Validation or reliability	Blinding of assessor
Sheikh 2004 [14707871]	MYCL.5NT	*L-MYC*	• Ratio of allele peaks <0.6 or >1.6	• FAL • 1 point for each of FAL>0.50; age>50; positive LN	Not stated	Not stated
	D1S407	*CMM*				
	D3S1539	*VHL*				
	D5S615	*APC*				
	D5S592	*MCC*				
	D9S252	*PTCH1*				
	D9S254	*MTS1/p16*				
	D9S251	*MTS1/p16*				
	D17S974	*p53*				
	D17S1289	*p53*				
	D22S417	*NF2*				
Maheshwari 2006 [17009159]	D3S2303 or D3S1539[o]	*VHL*	• Ratio of allele peaks <0.5 or >2, or beyond 2 SDs of the marker mean; • Unclear for *K-ras-2* point mutations	• FAL <25%, 25-50%, and >50%	Not stated	Not stated
	D5S592 or D5S615[a]	*MCC or APC*				
	D7S1530					
	D9S252	*PTCH1*				
	D17S974	*p53*				
	D17S1289	*p53*				
	"point mutations"[p]	*K-ras-2*				
Tse 2006 [16386976]	D1S407	*CMM*	• Ratio of allele peaks beyond 2 SDs of the marker mean	• FAL	Not stated	Blinded to clinical data
	D3S1539	*VHL*				
	D3S2303	*OGG1*				
	D5S615	*APC*				
	D5S592	*MCC*				
	D9S252	*PTCH1*				
	D9S254	*MTS1/p16*				
	D9S251	*MTS1/p16*				
	D10S520	*PTEN*				
	D10S1173	*PTEN(?)*				
	D17S974	*p53*				
	D17S1289	*p53*				
	D17S1161	*NME1(?)*				
	D22S417	*NF2*				
Finkelstein 2004 [15151207]	MYCL D1S407	*L-myc*	Not stated	• FAL	Not stated	Not stated
	D1S1193					
	D3S2303					
	D3S1539	*OGG1*				
	D5S592	*APC*				
	D5S615					
	D9S251					
	D9S254	*p16*				
	D10S520					
	D10S1173	*PTEN*				
	D17S974	*TP53*				
	D17S1289					
	D19S559					

Results

Table 3a, 3b and 3c summarize study findings.

Diagnostic accuracy (Level 2)

Two studies,[19,20] evaluated the diagnostic ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® to identified malignancy in Papanicolaou-stained smears from pancreaticobiliary cytology. Pathological confirmation of surgical specimens or long-term clinical followup was used as the reference standard.

In both studies, all samples with positive cytology had FAL>0. All samples with negative cytology had FAL=0 (with one exception in one study[20]). Using the above cutoffs, loss-of-heterozygosity based topographic genotyping with PathfinderTG® correctly classified all cases (10[19] and 11[20]) that were inconclusive with cytology analyses.

One study applied prospectively loss-of-heterozygosity based topographic genotyping with PathfinderTG® to distinguish reactive gliosis from glioma in 16 diagnostically challenging cases.[2] Sensitivity was 89% (8/9) and specificity 100% (7/7). However, these estimates are extremely uncertain (small sample): the confidence intervals range from approximately 50% to 100% for both sensitivity and specificity.

Prognostic accuracy (Level 2)

The remaining 12 publications evaluated the association of PathfinderTG® aggregate scores and survival, recurrence-free survival, and tumor recurrence. Retrospectively collected clinical data were used to ascertain outcomes.

Treatment was not used as a covariate in all but one study.[22] This was the only eligible study that presented multivariate analysis estimates. One additional study[21] specifically commented that multivariate analyses were not possible because of complete separation of the outcome categories by the predictor (FAL equal to or greater than 0.20). In all other studies, confounders of the relationship between FAL and clinical outcome were not reported or adjusted for.

Cutoff selection (for FAL) appears to have been selected post-hoc (cutoffs across studies ranged from 0 to 0.63, when reported). In nine publications FAL was identified as a statistically significant predictor of a clinical outcome in at least one analysis or subgroup and for the presented cutoffs.

In three studies there were no statistically significant associations between FAL and the examined clinical outcomes in any analysis or subgroup.[14,17,25]

Impact on diagnostic thinking (Level 3)

Here we are interested in whether using loss-of-heterozygosity based topographic genotyping with PathfinderTG® affects the ordering of additional diagnostic tests. No study provided relevant data. However, as mentioned above under “Diagnostic accuracy”, loss-of-heterozygosity based topographic genotyping with PathfinderTG® correctly classified 10 and 11 cases that were “inconclusive” with simple cytology alone in two studies.

Impact on treatment decisions (Level 4)

Here we are interested in changes in treatment decisions given the results of loss-of-heterozygosity based topographic genotyping with PathfinderTG® compared to treatment decisions in the absence of such information. No study provided relevant data.

Impact on patient outcomes (Level 5)

No studies evaluated whether the use of loss-of-heterozygosity based topographic genotyping with PathfinderTG® affects patient outcomes.

Table 3a. Study findings for gastrointestinal tumors.

Author Year [PMID]	Aggregate score cutoff	Outcome definition	Statistical analysis and results
Cong 2001 [11260864]	Not stated	Survival after surgery by groups based-on specific LOH loci	3-y survival by Group [KM] I: 0%, II: 89%, III: 30% (P=0.049 [II vs. III])
Finkelstein 2003 [12668980]	0.3 (median of the whole samples)	Recurrence-free survival (RFS) after hepatic transplant	5-y RFS by allelic loss [KM] • FAL > 0.3: 12% vs. 97% (P<0.0001) • Reclassified T-stage based on allelic loss (T4 vs. T3 vs. T2/1): 0% vs. 42% vs. 85% (P<0.02) Multivariate analysis Vascular invasion (P=0.001), largest tumor size (P=0.003), and FAL (P=0.001) were statistically significant independent risk factors to predict recurrence (Cox model, backward stepwise elimination)
Marsh 2003 [12827550]	Not stated	Recurrence-free survival (RFS) after hepatic transplant	Univariate survival analysis • 9/18 markers were statistically significant (P<0.05) by log-rank test Prognostic accuracy of FAL • 5/103 (5%) excluded • 44/103 (43%) inconclusive test • Sensitivity of 94% (17/18) • Specificity of 97% (35/36) Prognostic accuracy of ANN of common prognostic factors and FAL • 10/103 (10%) excluded • 12/103 (12%) inconclusive test • Sensitivity 100% (29/29) • Specificity 100% (52/52)
Khalid 2004 [15542529]	Not stated	Diagnosis of cancer (inferred)	Mean FMR • 0.45 for positive cytology vs. 0.38 for inconclusive cytology (NS) Diagnostic accuracy • Sensitivity of 100% (17/17) • Specificity of 100% (9/9) • Accuracy of 100% (26/26) Diagnostic impact • No changes in positive cytology • No changes in negative cytology 10 changes in inconclusive cytology (9 became [true] positive and 1 became [true] negative)
Khalid 2006 [17029619]	Not stated	Diagnosis of cancer (inferred)	Mean FAL • 0.52 for positive cytology vs. 0.47 for inconclusive cytology (NS) Diagnostic accuracy • Sensitivity of 100% (15/15) • Specificity of 83% (5/6) • Accuracy of 95% (20/21) Diagnostic impact • No changes in positive cytology Eleven changes in inconclusive cytology (5 became [true] positive, 1 became [false] positive, and 5 became [true] negative)
Schwartz 2008 [18602719]	0.27	Recurrence	Among all 70 patients, the cutoff of 0.27 in FAL (selected with an ROC for recurrence in the 70 patients) is a strong predictor of

Author Year [PMID]	Aggregate score cutoff	Outcome definition	Statistical analysis and results
			recurrence: • HR 39.7 (95% CI: 5.3, 299.5) adjusted for Milan criteria, AFP, and histology (grade, invasiveness status) • [sensitivity and specificity not reported] Among 35 patients beyond Milan criteria, FAL >=0.27 has • Sensitivity 83% • Specificity 91% Among 35 patients beyond Milan criteria, a model including FAL and macroscopic vascular invasion (unclear if it includes other adjustments): • Sensitivity 83% • Specificity 91%
Fasanella 2009 [19152901]	0.20	Mortality Recurrence (based on CT) Progression (based on CT)	Median follow up was 30 mo (range 2, 66 months) From survival analyses, using the 0.20 cutoff for FAL (ten patients had FAL≥0.20): • Survival: All eight deaths among those with FAL ≥0.20. Log rank p<0.001 • Progression or recurrence: All 10 events among those with FAL ≥0.20 (log rank p<0.0001).

AFP: alpha fetoprotein; CI: confidence interval; CT: computed tomography; FAL: fractional allelic loss; HR: hazard ratio; NS: not significant

Table 3b. Study findings for lung tumors.

Author Year [PMID]	Aggregate score cutoff	Outcome definition	Statistical analysis and results
Sasatomi 2002 [11980668]	0.63	Survival	P=0.552 median survival 3.3 y vs. 3.4 y (est.)
	0.50	Survival	P=0.012 median survival 3 y vs. >7 y (est.)
Sasatomi 2004 [15371943]	0.50	Survival	In bronchoalveolar carcinoma: favoring low FAL group, P=0.098
			In stage I adenocarcinoma: no correlation between LOH, maximum FAL and clinical outcome
Dacic 2005 [15958854]	0.4	Survival	P=0.159 median survival 59 mo vs. 10 mo
Fernando 2004 [14752417]	high risk (FAL_{node}/FAL_{tumor} ratio ≥1); low risk (ratio <1)	Survival	squamous carcinoma: median survival 38 mo (est.) vs. 34 mo (est.); NS adenocarcinoma: median survival 25 mo vs. no death; P=0.01

AFP: alpha fetoprotein; CI: confidence interval; CT: computed tomography; FAL: fractional allelic loss; HR: hazard ratio; NS: not significant

Table 3c. Study findings for other tumors.

Author Year [PMID]	Aggregate score cutoff	Outcome definition	Statistical analysis and results
Sheikh 2004 [14707871]	Not applicable	Tumor recurrence	P=0.08 Mean FAL: Recurrent (n=6): 52% Non-recurrent (n=5): 34%
	FAL>0.50	Tumor recurrence	P=0.10 High FAL: Recurrent: 4/6 Non-recurrent: 1/5
	>1 points	Tumor recurrence	P=0.004 2 or 3 points: Recurrent: 6/6 Non-recurrent: 0/5
Maheshwari 2006 [17009159]	FAL <25%, 25-50%, and >50%	Survival analysis	P<0.05 Worse FAL category is associated with worse survival
Tse 2006 [16386976]	Not stated	Survival [among 7 receiving conventional treatment]	P=0.15 No significant effect of FAL on time to death (direction of effects not reported)
	Not stated	Survival [all patients (stratified per therapy)]	P=Not stated No significant effect of FAL on time to death
	Not stated	Time to recurrence	P=0.15 (from 95% CI) HR 2.6 (95% CI: 0.7-9.3) per 20% increase in FAL
Finkelstein 2004 [15151207]	FAL>0	Diagnosis of reactive gliosis vs glioma	Sensitivity: 8/9, 89% (95% CI 52, 100%) Specificity: 7/7, 100% (95% CI 59, 100%)

AFP: alpha fetoprotein; CI: confidence interval; CT: computed tomography; FAL : fractional allelic loss; HR: hazard ratio; NS: not significant

Overview

All studies were retrospective in design and used archival tissue blocks that were available. In a single study, molecular profiles of gliomas and reactive gliosis were determined retrospectively, and then they were used *prospectively* on 16 diagnostically challenging cases of reactive gliosis versus glial tumors.[2]

Three publications examined the ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® to diagnose malignancy from pancreatico-biliary cytology specimens,[19-21] whereas all other studies used tissue slides from solid tumors.

Microdissection and molecular analysis protocols were described in detail in all studies. However, details on patient (sample) selection, patient characteristics, treatments received, clinical endpoint definitions, justification of sample size, selection of cutoffs (for aggregate scores such as FAL), selection among various statistical models, and other important parameters of study design and reporting were provided inconsistently. **Table 4** shows whether the assessed studies reported information suggested by the REMARK guidelines.

Table 4. Reporting of several characteristics across the eligible studies (items adopted from the REMARK statement).

	18	1	23	19	20	16	22	21	17	14	15	24	26	25	2
Clearly stated study objectives	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	N[17]	N	N
Described patient characteristics	Y	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
Described treatments	N	N	N	NA	NA	N	N	Y	N	N	Y	N	N	N[18]	N
Described biological materials	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Specified assay method	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Described method of case selection	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y	Y	Y	Y	N
Defined clinical endpoints	N	N	N	N	N	Y	N	N	Y	Y	Y	Y	Y	N[19]	N
Listed all variables in model a priori	N	N	N	NA	NA	N	Y	N	Y	Y	Y	N	N	N	N
Explained sample size choice	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Described statistical methods	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N[20]	N
Described methods for cutpoint determination	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N
Described patient flow	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N
Described demographics	Y	N	N	N	Y	Y	Y	Y	Y	Y	Y	N	N	N	N
Described relation of marker to standard predictors	N	Y[21]	Y[22]	NA	NA	Y	N	N	N	N	N	N	N	N	N
Presented univariable analyses	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y
Reported multivariate analysis estimates	N	N	N	N	N	N	Y	NA[23]	N	N	N	N	N	N	N
Provided estimates and confidence interval for all predictors	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Any internal validation	N	Y	Y	N	N	Y	N	N	N	N	N	N	N	N	N
Described limitations of predictive instrument	N	N	N	N	Y	N	N	N	N	Y	N	N	N	N	N
Described implications for future research	N	N	N	N	N	Y	Y	Y	Y	N	Y	N	N	N	N

N: No/Not stated; NA: not applicable; Y: Yes
Studies ordered as in **Table 1a, 1b, and 1c**.

Key Question 3: Is there direct evidence comparing loss-of-heterozygosity based topographic genotyping with PathfinderTG® with conventional pathology without such analyses for clinical outcomes?

We did not identify eligible studies for this question.

Key Question 4: Was informed consent and IRB approval obtained, and were institutional guidelines followed?

All studies except for two specifically mentioned that IRB approval was obtained for the specific study. Two studies[18,24] did not mention any information. However, during the peer-review period the company clarified that all studies were conducted according to institutional mandates.

We clarify that for retrospective studies using archival samples and retrospective chart review it is generally not mandated to obtain informed consent from patients. Typically, the IRB would provide approval as long as the protocol of the study is in agreement with the international and institutional ethical mandates.

Key Question 5: Applicability of studies to the Medicare population

None of the studies explicitly stated their applicability to the Medicare beneficiary population. Five studies did not provide any information on the age distribution of the included patients whose tumors were examined.[1,2,19,23,25] In the remaining eight studies, mean ages ranged from 48 to 70 years (above 60 years in six studies; **Table 1a**, **1b**, and **1c**).

The applicability of the findings to Medicare beneficiaries cannot be easily deduced for several eligible studies. This is further complicated by the relative dearth of information on the selection process of the samples that were included in the analyses.

Discussion

Most studies on loss-of-heterozygosity based topographic genotyping with PathfinderTG® were excluded because they only described the molecular profile of different tumors, without assessing the ability of the method to help make diagnosis, prognosis or treatment guidance. No studies directly measured whether using loss-of-heterozygosity based topographic genotyping with PathfinderTG® improves patient-relevant clinical outcomes. Eligible studies on the diagnostic and prognostic ability of loss-of-heterozygosity based topographic genotyping with PathfinderTG® were small in sample sizes and had overt methodological limitations. Important characteristics of their designs were not clearly reported. Most studies clearly reported receiving IRB approval. Evaluating the applicability of studies to the Medicare beneficiary population was hindered by the lack of details on patient characteristics.

Loss-of-heterozygosity based topographic genotyping with PathfinderTG® is claimed to be particularly useful in cases where conventional pathology is unable to provide a conclusive diagnosis. However, the included studies were not designed to address this question. (An exception is a single small study where loss-of-heterozygosity based topographic genotyping with PathfinderTG® was used prospectively in 16 patients with challenging differential diagnosis between reactive gliosis versus glioma.[2]) Therefore, it is unclear if the findings of the reviewed studies are directly applicable to patients with the same cancers but with inconclusive diagnosis.

Ultimately, the value of any diagnostic or prognostic test is determined by its ability to affect patient-relevant clinical outcomes. As of this writing, there are no studies –comparative or not– on whether patients who received loss-of-heterozygosity based topographic genotyping with PathfinderTG® testing had better survival, longer time to tumor recurrence, or fewer adverse outcomes attributable to unnecessary harmful interventions.

This systematic review identified several limitations of eligible studies on loss-of-heterozygosity based topographic genotyping with PathfinderTG®. First, all studies had very limited sample sizes and with one exception had performed only retrospective assessments. Although they provided details on pathologic and biochemical protocols, they did not provide important information on patient selection, patient characteristics, treatments received, clinical endpoint definitions, justification of sample size, selection of cutoffs (for aggregate scores such as FAL), and selection among various statistical models. There were strong indications that the selection of cutoffs in the aggregate score (FAL) was determined post-hoc: FAL cutoffs varied widely across studies (from 0 to 0.63) and were not validated in an external population (with a single exception of a prospective assessment of 16 cases). Finally, studies evaluating prognostic ability did not adjust for treatment or other predictors of outcome and did not provide multivariate analyses.

Allowing for the aforementioned caveats, it is theoretically and biologically plausible that topographic genotyping (including loss-of-heterozygosity based topographic genotyping with PathfinderTG®) may have prognostic and diagnostic ability, if one examines a suitable genetic marker panel for each type of cancer. The reviewed studies are suggestive of the above for the patented loss-of-heterozygosity

based topographic genotyping with PathfinderTG® methodology. However, all studies are small, they have important methodological limitations, and they do not address patient-relevant outcomes.

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