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REVIEW

Endoscopic biopsies from gastrointestinal carcinomas and their suitability for molecular analysis: a review of the literature and recommendations for clinical practice and research

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Endoscopic biopsies from gastrointestinal carcinomas and their suitability for molecular analysis: a review of the literature and recommendations for clinical practice and research.

Endoscopic biopsies (EBs) are the gold standard for diagnosing gastrointestinal carcinoma yet no guidelines address EB use for prognostic and predictive molecular testing. This review summarizes the reported quantity and quality of EBs, their relationship with molecular test failure rates and the resultant concordance between EB and resection specimen. Studies reporting molecular testing on gastrointestinal carcinoma EBs published between 2002 and 2014 were identified. Details regarding EB quantity, quality, tumour content, molecular test failure rates as well as causes and concordance with resection specimens were reviewed. Seventy-five studies were identified. Eighteen (24%) reported the mean EB number per patient (median: 2.1, range: 1–6.6 EBs). Sixty-one (81%) reported the frequency of test failure (median: 0%, range: 0–100%). Twenty-two (29%) investigated EB and resection specimen concordance (range: 0–100%). EB quantity and quality affected neither concordance nor failure rate. In summary, few studies currently report EB quantity, EB quality or EB and resection specimen concordance. Reliable molecular testing in EBs appears achievable, and can be representative of resection specimens. Concordance depends upon the testing methodology and biomarker heterogeneity within the tumour. To improve patient care, EB sampling, processing and reporting requires standardization and needs optimization for each biomarker individually.

Keywords: gastrointestinal carcinoma, endoscopic biopsy, molecular profiling

Introduction

Endoscopy of the gastrointestinal tract is a relatively low-risk procedure for the diagnosis of gastrointestinal malignancy.[1] Endoscopic biopsy (EB) material is used increasingly in routine clinical decision-making for analysing mutation status, DNA copy number status or protein expression in order to determine a patient's eligibility for a particular drug treatment. For example, in patients with metastatic gastric carcinoma, human epidermal growth factor receptor 2 (ERBB2, also known as HER2) status in the diagnostic biopsies is used to identify patients eligible for treatment with trastuzumab. In patients with colorectal carcinoma, the *KRAS* and *NRAS* mutation status is used to stratify patients for treatment with epidermal growth factor receptor (EGFR) targeting drugs. EBs are also used to discover potential new therapeutic targets and prognostic and

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predictive biomarkers for treatment stratification. Most importantly, EBs may be the only tumour material available from patients with metastatic disease.

Endoscopically obtained tissue from the gastrointestinal tract may vary substantially in size and quality. While there are international guidelines providing recommendations on the number of biopsies to be taken when diagnosing carcinoma in the gastrointestinal tract (Table 1), there are currently no recommendations regarding the required quantity and quality of EBs to perform molecular tests for prognostic or predictive biomarker evaluation. However, before applying the findings from molecular tests to EBs in routine clinical practice, it is paramount that researchers and clinicians alike fully understand the potential limitations of EBs, such as biopsy site, size and number as well as relative tumour content and tissue quality. These factors may influence significantly whether results from EBs are accurate and representative of the whole *in situ* carcinoma. Poor concordance of biomarker testing results between the biopsy and the resection specimen may result in patients being mismanaged and being either denied appropriate treatment or prescribed an inappropriate potentially harmful chemotherapy regimen.

In order to develop guidelines on molecular testing in EBs for clinical practice and research, this review aimed to assess what details are currently provided by investigators reporting results from molecular tests in EBs. In addition, this review evaluated the frequency and causes of molecular test failure on EBs considering, in particular, whether there is evidence of a relationship between the number, size and tumour content of EBs and the concordance between molecular results from biopsies and matched resection specimens.

Methods

A search of the Medline and Embase databases was performed using the search terms outlined in Supporting information, Box S1, restricting the search to full manuscripts of original studies on human tissue samples of epithelial gastrointestinal carcinomas published in English during a 12-year period (2002–14). The reference lists of the included studies were searched to identify further relevant studies. For a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram displaying the number of studies included at each stage of the review process see Figure 1.

The published study reports were analysed regarding the reported number, size, quality and tumour content of the biopsies, the site of the biopsies within individual tumours (e.g. edge, centre or random), the molecular testing methodology used and the fre-

Table 1. Recommended number of biopsies for diagnosis of oesophageal/gastric and colorectal carcinomas in different countries/regions

Country/region	Recommended number of biopsies	Ref.
Oesophageal/gastric carcinomas		
Pan-European (EURECCA guidelines)	No guidelines	–
Germany*	8 biopsies (10 if larger (undefined) lesion)	67
Japan	No guidelines	–
UK	6 biopsies from suspicious lesions	68
USA	6–8 biopsies	69,70
Colorectal carcinomas		
Pan-European (EURECCA guidelines)	No number provided	71
Germany	No number provided	72
Japan	No guidelines	–
UK	No number provided	–
USA	No number provided	73,74

EURECCA, European registry of cancer care.
*Guidelines for adenocarcinoma only, no guidelines for squamous cell carcinoma.

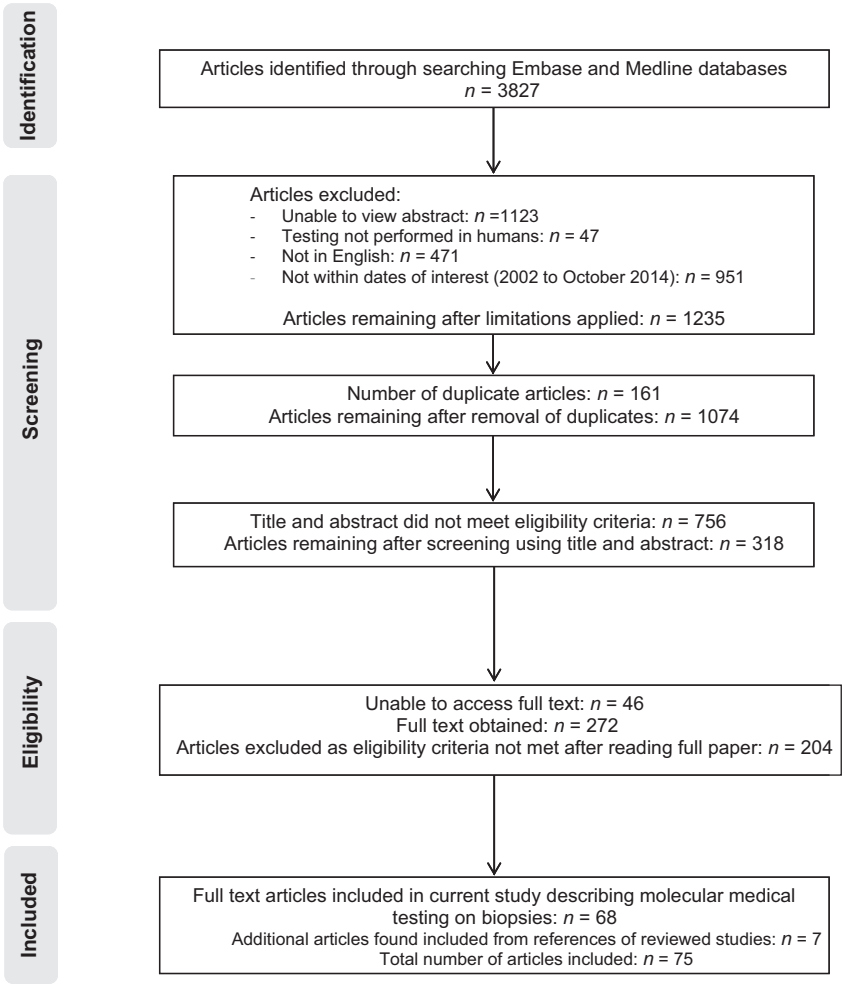

Figure 1. A Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram detailing the studies retrieved from the database search and the dropouts at each stage.

quency and suggested causes of molecular test failure. Furthermore, data on concordance of results between EBs and matched resection specimen was analysed.

STATISTICAL ANALYSIS

Descriptive statistics were calculated using Microsoft Excel 2010. Across all studies, the median and range number of patients and mean number of biopsies per patient were calculated. Where individual studies reported only the number of patients and the total number of biopsies, the mean number of biopsies per patient per study was calculated.

Results

QUANTITY AND QUALITY OF ENDOSCOPIC BIOPSIES

Seventy-five studies met the inclusion criteria (Figure 1). Twenty-five (33%) studies used biopsies from the colorectum, 47 (63%) reported biopsies from the oesophagus and/or stomach and three (4%) used biopsies from both the colorectum and the oesophagus/stomach. The median number of patients of all included studies was 47 patients (range: 5–574 patients). A variety of molecular tests was performed using either fresh, frozen or formalin-fixed paraffin-embedded (FFPE) EB tissue. These included *in situ* hybridization techniques, enzyme-linked immunosorbent assays, gene expression profiling, DNA sequencing, Western blotting, immunohistochemistry, gel electrophoresis, comparative genomic hybridization and mass spectrometry.

Eighteen (24%) studies reported the number of EBs used for molecular testing; the median (range) mean number of EBs per patient was 2.1 (1 to 6.6 biopsies).[2–15] Only one study reported the location within the tumour from which the biopsy was obtained.[16]

Eight (11%) studies included details on the size of the biopsies.[11,14,16–21] However, biopsy size reporting varied from being descriptive 'usually measuring

approximately 2–3 mm'[16] or 'approximately 2 mm in size'[17] to measurements of areas; mean area (1 mm^2),[18] median and range area (1.92 mm^2, 0.99–3.37 mm^2),[19] diameters (1–5 mm[14] and minimum diameter of 2 mm in greatest dimension[21]), length (mean length (1 mm))[20] and weight (weight range: 5–80 mg).[11] Only one study described how the reported measurements were obtained.[19]

The quantity of tissue used to perform the molecular testing varied among the different studies from requiring a certain number of sections to be cut from FFPE blocks, to numbers of cells or to specific quantities of DNA or RNA. Only 14 (19%) studies reported a minimum biopsy quantity or quality as a study inclusion criterion, each of them setting a different threshold (Table 2). Twenty-four (32%) studies provided no information regarding the quantity of material used to perform the test.[7,11,13,15,22–41] Among studies reporting RNA expression, up to 2 μg appeared to be extractable from one single fresh/frozen biopsy,[9,16] however, the authors reported neither the size nor relative tumour content of the biopsies used. None of the studies provided a rationale for choosing a particular quantity or quality.

The test failure rate was reported in 61 (81%) studies (median failure rate: 0%, range: 0–100% of tests). The reported reasons for test failure are summarized in Table 3.

CONCORDANCE OF RESULTS BETWEEN GASTROINTESTINAL ENDOSCOPIC BIOPSIES AND MATCHED RESECTION SPECIMEN

Twenty-two (29%) studies compared biomarker results from biopsies to those from matched resection specimens. Biomarkers were investigated by immunohistochemistry (IHC), gene expression studies, gene mutation analyses, *in situ* hybridization (ISH) or comparative genomic hybridization (CGH). In seven of the 18 studies the investigators detailed how they selected the region of interest from the resection specimen for the comparison, albeit without providing any rationale for this decision.[6,12,17,42,43] Four studies used one 'representative block',[17,35,42,44] one study used 'the most representative block, avoiding areas of ulcerative/necrotic phenomena',[12] one study used two to five sections of the largest representative cut surface[6] and one study used two samples from 'widely separated' areas without defining 'widely'.[43] Two studies reported a minimum tumour cell density required in the resection specimen for comparison using cut-offs of 10%[45] or 50%[54] tumour cells.[45] No association was seen among studies between the mean number of biopsies obtained per patient and the concordance of biomarker testing between biopsy and resection specimen.[2–4,6,7,9–15,20,25,35,36,42,46,47] Results for the different investigative methodologies are detailed below.

IMMUNOHISTOCHEMISTRY

In oesophageal squamous cell carcinoma (OeSCC), 80.7% concordance between biopsy and resection specimen was reported for cytokeratin 18 IHC using a binary score (≥50 or <50% positive carcinoma cells).[25] A concordance rate ranging from 50 to 88.5% between gastric carcinoma biopsies and resection specimens was reported for receptor tyrosine–protein kinase ERBB2 expression,[6,12,13,26,35,48–50] where overexpression was determined using the criteria described by Hofmann *et al.*[51] Relatively high kappa agreement values were reported for IHC scoring of vascular endothelial growth factor (VEGF) ($\kappa = 0.77$) and CD44v6 ($\kappa = 0.79$) using a binary score ('positive' being defined as moderate intensity staining in >1% of carcinoma VEGF and >25% of carcinoma cells for CD44v6 in at least two of three tissue microarray cores).[4] Eighty-two per cent concordance was reported for ATM expression between biopsy and resection specimen when using IHC and a binary score of positive (≥10%) versus negative (<10%) ATM staining in tumour cells. A much lower concordance rate was reported in gastric carcinoma when the staining intensity of EGFR (38% concordance)[6] or dihydropyrimidine dehydrogenase (DPD; 53% concordance)[17] were scored.

In colorectal cancer (CRC), a high concordance rate (≥95%) was shown for immunohistochemical staining of mismatch repair proteins MLH1, MSH2, MSH6, PMS2 in two independent studies. All four proteins were scored as either positive or negative, irrespective of the intensity of the staining, in one study and scored as positive, focal/weak or negative staining in the second study.[42,52]

Although TP53 was reviewed in both gastric and colorectal carcinoma, and the agreement between EBs and resection specimen was much higher in gastric carcinoma ($\kappa = 0.94$ versus $\kappa = 0.22$),[4,10] however, the reported kappa values are not directly comparable as the threshold for scoring TP53 'positive' differed between the studies.

GENE EXPRESSION PROFILING

In OeSCC, a study of 40 patients reported that 129 and 136 genes of the 18 734 investigated genes were

Table 2. The reported minimum quantity and quality of tissue required by studies which specified inclusion criteria before performing molecular testing

Carcinoma type	Molecular test method	Reported required tissue quality and quantity	Ref
OeAc	RT–PCR	At least two biopsies per case and a minimum of 2000 cells in total	53,75
OeAc	RT–PCR	>70% tumour content	24
OeSCC	IHC	Analysis region must not include areas of inflammation, sclerotic tumour or adjacent benign tissue and tumour area >0.6 mm^2, equivalent to three ×400 measurement fields	19
Gastric	IHC	No edge or crushing artefacts	49
Gastric	IHC	Five representative fields; each with 100 cells per area (500 cells total)	76
Gastric	IHC	Five representative fields; each with 200 cells per area (1000 cells total)	77
Gastric	ISH	mRNA must have first been verified as not degraded using a poly d(T)20 probe	55
Gastric	Gene expression array	RNA extraction performed only on samples which consisted of 100% carcinoma	16
Gastric	MALDI mass spectrometry	≥75% tumour cells (or normal cells where normal cells being analysed)	
CRC	IHC	'Well-preserved tissue' only	78
CRC	PCR and DNA sequencing	≥50% tumour cells	54
CRC	PCR and DNA sequencing	≥10% tumour cells	45
CRC	PCR and DNA sequencing	Tumour at least 2 mm in greatest dimension on a 10 μm unstained slide to derive a minimum of 6ng/μl of tumour DNA. Also, >30% of the sample must consist of viable tumour nuclei and <30% necrosis	21
Rectal	Gene expression profile	≥70% tumour cells	79

OeAC, Oesophageal adenocarcinoma; OeSCC, Oesophageal squamous cell carcinoma; IHC, Immunohistochemistry; ISH, *In situ* hybridization; PCR, Polymerase chain reaction; RT–PCR, Reverse transcriptase–polymerase chain reaction; CRC, Colorectal carcinoma; MALDI, Matrix-assisted laser desorption ionization time-of-flight.

Table 3. Reported causes of molecular test failure using gastrointestinal endoscopic biopsies

Problem	Number of studies reporting problem	Percentage of studies reporting problem	Reference
Admixture of normal epithelial or stromal tissue with neoplastic DNA	3	9	9,11,43
Heterogeneity*	17	50	6,10–13,17,26,35,42,43,45,49,52,80–83
Inadequate DNA/RNA quality	3	9	7,55,80
Insufficient DNA/RNA quantity	5	15	27–29,80,82
Insufficient tissue from biopsy	9	26	3,4,14,16,37,49,78,84
Poor biopsy quality	2	6	35,85
Unknown	2	6	18,23

*Heterogeneity was defined as the expression of the investigated markers in some but not all the biopsies taken, or in only some regions of the resection specimen.[12]

up-regulated in the resection and the matched biopsy specimens, respectively, of which 85 (66% of 129) genes overlapped.[9] Additionally, 518 and 506 genes were down-regulated in the resection and matched biopsy specimens, respectively, of which 444 (88% of 506) genes overlapped.[9] No information was provided regarding these genes, and thus it is unclear whether the discordance between biopsy and resection specimen was seen across all genes or only genes with a specific function.

In a study of oesophageal adenocarcinoma (OeAc), the median expression of *ABCC1*, *ERCC1* and *ERBB2*, measured by reverse transcriptase–polymerase chain reaction (RT–PCR), was significantly higher in the biopsy than in the matched resection specimen in all patients ($n = 38$); however, the absolute difference in expression was not stated.[53] Interestingly, the relative expression of genes between the biopsy and resection specimen remained constant and thus, the authors speculated that higher gene expression in the biopsies may be due to delay in fixation or longer fixation of the resection specimen.[53]

QUANTITATIVE PCR AND DNA SEQUENCING FOR MUTATION DETECTION

High kappa agreement (median κ: 0.89, range: 0.62–1) was seen between biopsy and matched resection specimens from colon carcinoma patients for loss of heterozygosity, determined by quantitative PCR (qPCR) or by direct sequencing of *APC*, *DCC* and *KRAS* genes and for microsatellite instability (MSI).[43] A concordance of >97% between biopsy and matched resection was seen for mutation testing of *KRAS*, *BRAF*, *PIK3CA* and *TP53* genes and MSI in colorectal carcinomas irrespective of the methodology used.[45,54] Furthermore, 100% concordance was demonstrated for the detection of both, the presence of a *KRAS* mutation and the location of the mutation site in a study of 30 CRC patients (12 of whom exhibited KRAS mutations) in the biopsy and matched resection specimens.[21]

IN SITU HYBRIDIZATION

In situ hybridization (ISH) was compared between biopsy and matched resection specimens in gastric carcinoma only. ISH demonstrated >80% concordance for *ERBB2* gene copy number assessment in four studies,[6,12,13,26] and a concordance of 62.5% in one study.[48] In three studies, the concordance between biopsy and matched resection specimen cannot be assessed, as ISH was performed either only on equivocal cases, giving a skewed reflection of its accuracy, or the agreement between biopsy and matched resection specimen was reported only for combined IHC and ISH.[44,50] Among studies investigating *ERBB2* copy number by ISH, four used the same criteria to define *ERBB2* amplification [ERBB2/chromosome 17 centromere (CEP17) ratio of ≥2.2 in 20 tumour cell nuclei or a ERBB2/CEP17 ratio of ≥2 in 40 tumour cell nuclei where the ratio in 20 nuclei lay between 1.8 and 2.2],[26,35,44,50] whereas all other studies used different criteria.[6,12,13,48] ISH was also used to investigate *EGFR* copy number in gastric carcinoma.[6,55] The concordance rate between biopsy and matched resection specimen for *EGFR* status varied by up to 30% in 80% of patients in one study of

189 patients[55] and was 0% in a much smaller second study of 14 patients.[6]

COMPARATIVE GENOMIC HYBRIDIZATION

Comparative genomic hybridization has been investigated in only one study of eight patients with OeSCC, in which 100% concordance between biopsy and matched resection specimens was reported for DNA copy number aberrations.[8]

Discussion

Molecular testing on endoscopic biopsies (EBs) of gastrointestinal carcinomas is becoming increasingly prevalent, and yet no recommendations exist regarding the amount of tissue necessary for accurate and reliable molecular testing. The results from our literature review indicate that a number of factors need to be considered when developing recommendations regarding the amount of tissue necessary for accurate and reliable EBs molecular testing.

First, there are technical factors related to the type of molecular assay performed, the minimum amount of tumour tissue/DNA/RNA required, the potential yield and quality of tumour tissue/DNA/RNA that can be obtained from a 'typical' EB and whether the assay requires fresh/frozen tissue or can be performed using FFPE material. The literature review finding that there was no difference in test failure rate using different quantities of biopsy tissue suggests that test failure was not related strictly to the amount of available tissue but was more likely related to qualitative factors (tumour content, fixation time, investigative methodology used). Therefore, simply obtaining more of the same tissue may not reduce the test failure rate. From the current literature review, the 'required' amount appeared to be one to two biopsies for gene expression profiling and DNA/RNA-based assays.[3,7,9,11,15] However, guidance has also been published; for example, ERBB2 IHC/ISH testing in biopsies that does not refer to a minimum number of biopsies, but the presence of a minimum of five cohesive cells, without providing a rationale for this.[49]

The second factor to consider is whether the biopsy material is representative of the *in situ* tumour. Understanding the potential causes of discordant results between biopsy and matched *in situ* tumour is essential, as biopsy results may lead otherwise to potentially inappropriate treatment decisions. The potential causes of discordant results include intratumoral biomarker heterogeneity, the testing method used, the FFPE process and the method of specimen selection for comparison.

Given the intratumoral heterogeneity described in the literature for some biomarkers in gastrointestinal carcinomas,[23,47] it seems logical that a greater number of biopsies should increase the chance of a biomarker being detected if present. The lack of association between an increasing number of biopsies and increasing biomarker concordance between biopsy and resection specimens in the published literature may either reflect that there have been only a small number of studies published assessing the concordance between biopsy and resection specimen, or may suggest that biopsies might need to be taken from a particular part of the tumour as simply taking more 'random' biopsies, does not seem to increase biomarker detection. Future research is needed to investigate whether there is a potential preferential spatial distribution of the biomarker of interest in a given tumour requiring 'targeted' biopsies (e.g. tumour edge versus centre versus 'random' region), or 'targeted' regions of the resection specimen for comparison by the investigator[6,56] instead of 'random' biopsies. Current national and international guidelines specify neither a biopsy site within a given tumour nor any adjustment of the number of biopsies in relation to the size of the tumour. Preferential expression of biomarkers at the invasive tumour edge, a part of the tumour that is inaccessible endoscopically for all but intramucosal carcinomas, has been demonstrated in both oesophageal and gastric carcinomas for several biomarkers in the past;[17,57–61] systematic assessment of the spatial distribution of any newly proposed biomarkers (lumenal versus deep tumour edge as well as within the lumenally accessible aspect) has not been performed so far.

The test method itself may be the cause of poor concordance between biopsy and matched resection specimen via the use of different scoring systems. In particular, review of the literature showed that the higher concordance rate between gene copy number determination using fluorescence *in situ* hybridization (FISH) compared to protein expression assessment using IHC may be related to the subjectivity of assessing staining intensity, staining location and estimation of the percentage of positively stained cells compared to objectively counting the number of signals in a predefined number of nuclei.[3,6,12,26] Furthermore, we noted that the simpler the scoring system, the higher the concordance between biopsy and resection specimen. Thus, assays with binary results such as absence or presence of a mutation demonstrated a much higher concordance between

Box 1: Proposed minimum dataset for the reporting of endoscopic biopsies from gastrointestinal carcinoma patients for biomarker testing

Items for endoscopists to report:

- Size of the lesion/tumour.
- Site of the biopsy within the tumour (tumour edge/tumour centre/ulcer rim/ulcer centre).
- Number of biopsies from each site, ideally with submission of biopsies from different sites in different containers.

Items for pathologists to report:

- Number of biopsies and site of biopsies received.
- Number of biopsies and site containing tumour.
- Size of tumour containing biopsy (using the largest diameter (mm) measured at the time of microscopy using for example the Vernier scale).
- Tumour content (estimated as biopsy area comprised of ≤25%, 25 to ≤50%, 50 to ≤75% or >75% tumour cells[86]).
- Proportion and total amount of material used for molecular testing.
- Diagnostic category of the biopsies (e.g. dysplasia and grade/unequivocal carcinoma/suspicious for carcinoma).
- Method of tissue processing (fresh/frozen/paraffin embedded material).
- Time between tissue removal (biopsy and/or resection) and formalin fixation (if known).
- Method of molecular assay(s), including controls.
- Method of assessment (scoring system used).
- Number of test failures/repeats among the study cohort and the suspected or proven reason for these failures.
- Any evidence of heterogeneity of biomarker status among the assessed biopsies and the heterogeneity present among different sites within the same tumour.

biopsy and resection specimen than assays yielding a spectrum of results, suggesting that the potential influence of tumour heterogeneity on the concordance of results between biopsy and resection specimen may also depend upon the testing methodology providing an explanation for a higher concordance of DNA/RNA assessment-based results compared to IHC studies.

Finally, discordance between FFPE biopsies and resection specimens may be due to temporal differences in the fixation and paraffin-embedding process. Tissue exposure to cold and ischaemia is well recognized to modify tissue immunohistochemical staining results, RNA/DNA yield and DNA/RNA quality.[62–65] While biopsies fix rapidly due to their small size, the fixation duration of large resection specimens is usually 48 h,[66] excluding transport time of the specimen from theatres.

Conclusions

The quantity and quality of endoscopic biopsies used for molecular testing and their concordance with testing results from matched resections is poorly reported in the current literature.

Discordance between biopsy and resection specimens may be due to the testing methodology used, inappropriate selection of biopsy or resection specimen regions for comparison and variation in the spatial distribution of different biomarkers, which may be different for different tumour types.

Given the increasing clinical need for reliable biomarker testing in endoscopic biopsies to inform treatment decisions, it is timely to propose a minimum data set for gastrointestinal carcinoma endoscopic biopsy reporting for biomarker research studies. Such studies will inevitably require close collaboration between pathologists, endoscopists and researchers to facilitate current and future molecular studies (Box 1). Standardized reporting will enable the systematic investigation of the spatial variation of biomarker expression within the tumour, enable the selection of the optimal testing method and provide guidance on the required biopsy tumour content for reliability of biomarker detection. These data will aid determination of the feasibility for each potential new biomarker individually, increase research study comparability and reproducibility and enable pathologists to emphasize the potential limitations of biopsy-based biomarker results when communicating with clinicians to determine treatment regimens.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Box S1. Search strategy.