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ABOUT COVER

AIMS AND SCOPE

The primary aim of the *World Journal of Gastrointestinal Pathophysiology* (*WJGP*, *World J Gastrointest Pathophysiol*) is to provide scholars and readers from various fields of gastrointestinal pathophysiology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGP mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal pathophysiology and covering a wide range of topics including disorders of the esophagus, stomach and duodenum, small intestines, pancreas, biliary system, and liver.

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FRONTIER

Molecular and genetic markers in hepatocellular carcinoma: *In silico* analysis to clinical validation (current limitations and future promises)

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Abstract

Hepatocellular carcinoma (HCC) is the second cause of cancer-related mortality. The diagnosis of HCC depends mainly on α-fetoprotein, which is limited in its diagnostic and screening capabilities. There is an urgent need for a biomarker that detects early HCC to give the patients a chance for curative treatment. New targets of therapy could enhance survival and create future alternative curative methods. *In silico* analysis provides both; discovery of biomarkers, and understanding of the molecular pathways, to pave the way for treatment development. This review discusses the role *of in silico* analysis in the discovery of biomarkers, molecular pathways, and the role the author has contributed to this area of research. It also discusses future aspirations and current limitations. A literature review was conducted on the topic using various databases (PubMed, Science Direct, and Wiley Online Library), searching in various reviews, and editorials on the topic, with overviewing the author's own published and unpublished work. This review discussed the steps of the validation process from *in silico* analysis to *in vivo* validation, to incorporation into clinical practice guidelines. In addition, reviewing the recent lines of research of bioinformatic studies related to HCC. In conclusion, the genetic, molecular and epigenetic markers discoveries are hot areas for HCC research. Bioinformatics will enhance our ability to accomplish this understanding in the near future. We face certain limitations that we need to overcome.

Key Words: Hepatocellular carcinoma; *In silico* analysis; Bioinformatics; Biomarkers; Molecular pathways; Genetics; Epigenetics

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Core tip: Hepatocellular carcinoma (HCC) is the second cause of cancer-related mortality. The importance of having an early detecting biomarker is to allow for curative measures to be applicable, and prognostic biomarkers to detect survival, in dealing with the disease. *In silico* analyses allow us to discover new genetic and epigenetic biomarkers, along with establishing the coexpression patterns, which impact HCC survival. Also, it allows for understanding the molecular pathways for HCC pathogenesis, and the discovery of potential therapeutic options. In this article, I review the current discoveries and limitations that face researchers to reach their ultimate goal of establishing clinical practice guidelines. I give an overview of the future potential that could benefit integrated research on HCC and discuss my own research related to the topic.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. HCC is the sixth most prevalent cancer, and the second leading cause of cancer mortality[1]. The incidence of HCC in different countries is related mostly to the geographic prevalence of certain risk factors such as chronic hepatitis B and C, aflatoxins, and alcoholism[2,3]. HCC causes annual morality exceeding 700 000 cases[4], and high recurrence rate after treatment, with overall 5-year survival (< 50% of cases)[5], and even lower numbers reach the endpoint of 10-year survival (< 10% of cases), despite aggressive treatment[6].

Cirrhosis can proceed to HCC in 5%–30% of patients after an average duration of 5 years[7]. Most HCC cases arguably occur on top of cirrhosis, but we cannot ignore the 20% of cases that occur without any preceding cirrhosis. Thus, cirrhotic and noncirrhotic causes of HCC are explained by different pathogenic mechanisms[8,9].

A debate has arisen about the relation of increased incidence of HCC among patients receiving new direct-acting antivirals (DAAs) for hepatitis C treatment, but recent studies have shown that the risk of *de novo* and recurrent HCC with DAAs is actually lower than without, although not completely abolished[10,11].

Many HCC studies use bioinformatics as a method to determine the molecular pathways affected by HCC, along with the genetic and epigenetic control of those pathways. These proteomic and genomic studies are the future of personalized medicine, where precision therapy could offer patients a management plan specified for their individual mutations, with high curative capabilities. We have visualized how intercepting a molecular pathway as in sorafenib, a multikinase inhibitor that enhances apoptosis, could increase the survival of advanced HCC by several months, but unfortunately, recent studies have shown that resistance to the drug is evolving [12,13].

The Cancer Genome Atlas Research Network has conducted a study project on 33 cancers with poor prognosis, provided that there were suitable available tissue samples for experimental validation through antibodies' multiplex analysis, and they included HCC among other cancers. The HCC study included 363 cases for whole-exome sequencing and 196 for further proteomic, epigenetic and DNA-methylation analyses. They found that the molecular pathways most affected in HCC are those that deal with the following: cell proliferation, differentiation, growth, apoptosis, and immortalization (through telomerase)[14].

In this review, I explore the steps for validation of molecular markers, with the limitations encountered to validate a novel biomarker, the research in molecular pathways related biomarkers, the role of bioinformatics in the discovery of those pathways, and future aspirations.

IMPORTANT QUALITIES IN DIAGNOSTIC AND PROGNOSTIC MARKERS THAT HAVE TO BE MET

The early diagnosis of HCC is a crucial issue, as all of the available curative measures are only effective in early stages of cancer (liver resection, liver transplantation, radiofrequency ablation). They are curative in early the Barcelona Clinic Liver Cancer stages (0 and A), where the size of the tumor does not exceed 5 cm in its largest diameter in one nodule, or the size does not exceed 3 cm in three nodules (Stage A) [15]. Thus, early screening of HCC is an effective tool for both early detection and treatment, which increases overall survival and yields better prognosis. Unfortunately, the screening process of HCC suffers a huge limitation, which is the low sensitivity of its most accepted biomarker, α-fetoprotein (AFP).

So, the current European Association for the Study of the Liver (EASL), and the American Association of Study of Liver Disease (AASLD) guidelines recommend the following; due to cost-effectiveness, abdominal ultrasound should only be used in the screening process, while AFP is limited to the diagnosis or screening of at high-risk populations. Where we find AFP sensitivity reaching as low as 20% positivity in early stages of cancer, with fluctuating levels of the biomarker in cirrhotic patients due to other reasons, causing further confusion in reaching the diagnosis[16-18], AFP is removed from the screening assessment guidelines altogether, as mentioned above.

It is important to note that, in Japan, the at-risk populations for HCC are still screened by 3-mo abdominal ultrasound, and AFP, in addition to another two biomarkers, *lens culinaris*-agglutinin-reactive fraction of AFP, and PIVKA-II (protein-induced by vitamin K absence or antagonist-II). All these are included in the Japanese insurance plan of at-risk populations[19]. Other markers considered for HCC diagnosis are: Dickkopf-1, which is a good biomarker for HCC with negative AFP[20], and des--carboxy prothrombin, which is directly correlated with tumor size and has higher sensitivity than AFP, so can be used in screening more effectively[21]. Unfortunately, none of the aforementioned biomarkers reaches the final acceptance to be added to any of the clinical practice guidelines for HCC due to cost-effectiveness, difficult availability, or high variability across studies.

I shared in the research work of determining some of the cost-effective biomarkers that are both cheap and effective, for establishing the diagnosis and staging of HCC, including a study on Golgi protein (GP)73, where the combined sensitivity of both AFP and GP73 was 84.4% and specificity of 95.6%[22]. Our results were similar to the meta-analysis of the diagnostic accuracy of GP73 in HCC, where combined GP73 and AFP had pooled sensitivity of 87% and pooled specificity of 85%[23].

AFP is the only widely validated biomarker for HCC diagnosis, and prognosis in most clinical guidelines despite its limitations. To overcome its limitations we are still searching for a new biomarker. This is an ongoing process, requiring computational, experimental, and clinical validation. Figure 1 shows the most important factors that are required in an effective diagnostic and prognostic biomarker.

STEPS FOR VALIDATION FOR A NEW BIOMARKER

The only approved biomarker for diagnosis by both the American and European guidelines is AFP. Other biomarkers are approved in Japanese and Korean guidelines as mentioned earlier. AFP, the biomarker that stood the test of time, has its own problems as low sensitivity making it weak as a surveillance method, which caused the ASLD and EASL to remove it from the screening of HCC except for high-risk populations[24,25]. Searching for a new biomarker is an ongoing process, which needs computational, experimental, and clinical validation, as shown in Figure 2.

The novel biomarkers discovered through bioinformatics analysis usually pass through different steps of validation. First, computational validation *(in silico* validation), through assessing correlated genes, then by statistical analysis of different genetic expressions[26]. Hence, the most statistically significant biomarkers, with a plausible molecular pathogenic background, will pass to the next stage. Experimental validation on the HCC tissues on resected tumor from patients or experimental laboratory cells as HeLa cells (*in vitro* studies). Later, clinical validation in the sera of patients with established diagnosis to determine the actual *in vivo* predictive diagnostic and prognostic capabilities of the biomarker. In this stage, we calculate the diagnostic test accuracy of the biomarker through identifying its specificity, sensitivity, and area under the curve (AUC), along with other important related parameters.

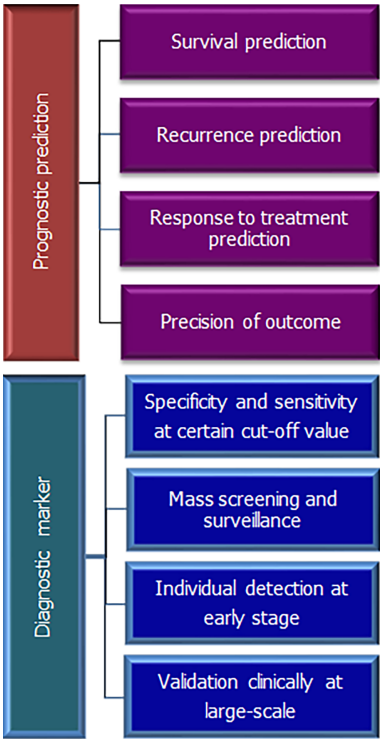

Figure 1 Showing important features of prognostic and diagnostic markers.

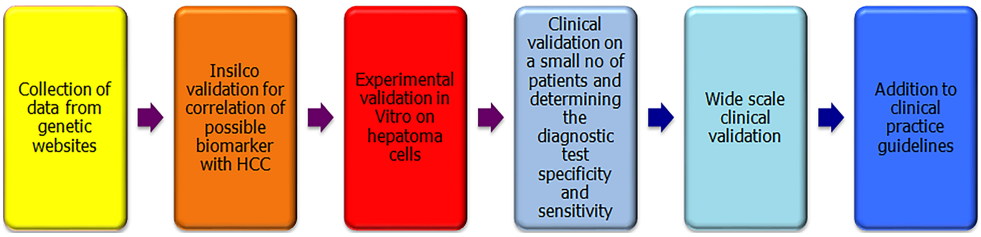

Figure 2 Pathway for validation of the biomarkers in hepatocellular carcinoma.

Diagnostic biomarkers should have a correlation with a clinical endpoint to be used in clinical trials, whether to help assessing this clinical endpoint or relate to it, as a surrogate marker. Then, external validation is examined through wide-scale studies, for the most acceptable biomarker in sensitivity and specificity. These studies must be done on variable and random populations (different ethnicity, gender, age groups, stages of the disease, *etc.*). When the biomarker reaches this stage of diagnostic accuracy validation, and proves to be cost-effective, then it can be added to clinical practice according to the level of evidence provided (the type of studies conducted *i.e.*, cohort, randomized controlled trial, case–control, *etc.*, and the size of the population examined)[27-29].

The steps for validation of a miRNA biomarker are[30]: (1) Data processing and screening of differentially expressed miRNAs; (2) Construction of the miRNA signature; (3) Confirmation of the miRNA signature; (4) miRNA signature validation using the OncomiR database and Gene Expression Omnibus (GEO) dataset; (5) Functional analysis; (6) *In vitro* analysis; (7) Testing on patients sera for diagnostic test accuracy; (8) Wide-scale clinical validation; and (9) Adding as a biomarker to the HCC diagnosis guidelines according to the level of evidence.

DISCOVERING NEW BIOMARKERS: MOLECULAR PATHWAYS DISCOVERY AND DETERMINING OF THE GENETIC-EPIGENETIC-PHENOTYPIC LINKAGE THROUGH CLINICAL STUDIES

HCC is a cancer of poor prognosis, especially when discovered in late stage, which is usually the case, due to lack of early detection by biomarkers, lack of effective chemotherapeutic treatment, and limited molecular target treatment. Understanding the molecular and genetic pathways is vital to overcome these obstacles, and reach better prognostic outcomes. Recently, an accumulation of data regarding genetic and epigenetic biomarkers became available, for both *in vitro* laboratory analysis and *in silico* analysis[31].

Many pathways have been critically assessed as the key element of early diagnosis of HCC or as the key predictive outcome (whether metastasis, relapse or complete recovery) after curative interventions. These pathways include cellular effects, such as: cell proliferation, growth, differentiation and immortality. Moreover, disturbance or mutation affects functions such as: vascular angiogenesis, inflammatory response, programmed cell death and autophagy. Formulation of drugs that could intercept these molecular pathways to establish a treatment plan with good prognostic capabilities is under investigation[32].

Autophagy pathway in HCC: published and unpublished work of the author

Autophagy is defined as the degradation of cellular components by lysosomal fusing with autophagosomes, and forming autolysosomes, as a homeostatic regulation for aging, stress, immunological response, or anticancer response. The role of autophagy in HCC is a complicated one. Whereas basal autophagy is responsible for anticancer protection of the organ, as carcinoma progress to a late stage, autophagy helps the cancerous tissues' survival and growth. Autophagy genes and their regulatory proteins linked to HCC include, *Beclin-1*, *ATG5* and *ATG7*. They control many molecular pathways such as: phosphatidylinositol-4,5-bisphosphate 3-kinase PI3K/AKT/mTOR, ERK/mitogenactivated protein kinase (MAPK), and apoptosis p53 pathways among others[33-36].

Our research on this pathway, linking the autophagy control of *ATG-4B* mRNA expression through noncoding miRNA-661 through bioinformatics methods proved to be of a clinical value after clinical validation. We found that combination of both biomarkers had specificity of 82.1% and sensitivity of 100%, especially in early HCC. The prognosis in the form of tumor-free survival was improved with the decline in the serum level of the two biomarkers as proved by multivariate analysis[37].

Hepatitis B and C associated HCC and the molecular pathways discovered: published work

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are the most common risk factors associated with HCC[38]. HBV is responsible for about half of HCC cases worldwide, in addition to most of the childhood associated HCC[7].

In a recent study, the researchers performed bioinformatics analyses using data from GEO database, to show the possible molecular pathways, which cause HBV to induce HCC. They formed heatmaps of the top 50 downregulated genes, and the top 50 upregulated genes associated with HCC occurrence. They found that there are six genes most significantly controlling the following pathways: carbon, certain amino acids, and retinol metabolism. They presented the molecular and cellular cycle pathways through the protein–protein interaction networks[39]. Furthermore, HBV-related HCC is linked to mutation of the *TP53* gene, along with viral genetic integration with the host DNA[14].

As for HCC associated with HCV, while using hierarchical clustering of the hub genes[40], the authors found overexpression of three genes: cyclin B1 coding gene, kinesin family member 20A coding gene, and hyaluronan mediated motility receptor coding gene. These were associated with decreased survival in patients with HCV-associated HCC[41].

Similarly, our team conducted a study about the relation of *IL-28* genetic polymorphism and HCC associated with HCV, in the era of interferon treatment of HCV infection. We found that the T allele was higher in both chronic liver disease (CLD) and HCC groups, with prevalence of 50% and 70%, respectively. As compared to the C allele, where the prevalence in CLD *versus* HCC groups was 30% and 50%, respectively, but the differences between the groups were not significant[42]. Our results were similar to a recent study conducted on the Chinese population, which

found that the T allele was associated with a higher risk of HCV-related HCC[43]. A recent meta-analysis found a strong correlation between *IL-28B* genetic polymorphism and HCC association with HBV or HCV infection, where CC and TT genotypes of certain single nucleotide polymorphisms (SNPs) of *IL-28B* were protective against HCC occurrence[44].

In addition, a bioinformatics study found that the *IL-28B* gene has a relation to HCC recurrence through gene expression profiles on 20 HCC *versus* 91 CLD samples, further researched *in silico* by gene set enrichment analysis and one-way hierarchical clustering for microarray analysis. They found on subsequent clinical validation in 183 HCC patients that certain *IL-28B* locus SNPs are associated with HCC recurrence [45].

Role of noncoding RNAs as epigenetic biomarkers for HCC: including published work

Both long noncoding RNAs and miRNAs are considered noncoding chromosomal regions, originating in the introns of the chromosomal DNA. They are responsible mainly for the control of the exons' stimulation–inhibition process[46]. Exons are the chromosomal blocks of DNA responsible for encoding proteins. The noncoding RNAs have many functions including: controlling protein metabolism, and maintaining chromosomal structure, besides segregation through telomerase generation[46,47].

As an example, the role of miRNA-20a in controlling the mRNA of *CyclinD1* (a proto-oncogene) was studied using bioinformatics prediction methods through matching the seed region of the miRNA with the chosen sequence, to predict related miRNA targets. This oncogene is responsible for controlling progression of G1 to S phase, and hepatocellular growth, through regulation of the Wnt signaling pathway. Later, this was confirmed by experimental validation in HepG2 cells[48]. Table 1 shows the molecular pathways.

Also, miR-1180-3p is upregulated in HCC and associated with increased tumor proliferation, resulting in poor survival. Functional computational analysis and KEGG pathways maps showed that this epigenetic marker is linked to MAPK pathway regulation, in addition to control of cellular proliferation, apoptosis and differentiation [49]. Table 1 shows the molecular pathways.

Our team has published work on the relation between different miRNAs and oncogenes and HCC, especially those associated with HCV, and comparing their diagnostic efficacy to that of the established marker AFP. For example, autophagy genetic markers are correlated with miRNA-661, as mentioned earlier. lncRNA-UCA1 (urothelial carcinoma associated 1), c-JUN (cellular jun proto-oncogene), miR-143 and miR-550a were studied in the serum of HCV-infected HCC patients[37,50,51]. lncRNA-UCA1 had a sensitivity of 91.4% and specificity of 88.6%, while c-JUN had a sensitivity of 91.4% and specificity of 91.4% with AUC of 91%[51]. Also, miR-550a had an inverse relation with miR-550a with sensitivity of 91.89% and specificity of 90.24%, while miR-143 did not show any relation to HCC occurrence[50].

Role of telomeres in HCC initiation and prognosis: work in progress

Telomeres function mainly in capping the chromosomal end to protect it from damage. They consist of nucleoprotein repeats. Telomeres can be transcribed into long noncoding RNAs, thus having an epigenetic control on the telomere homeostasis and telomerase enzymatic activity. Telomeres that bear such functions are called telomeric repeat-containing RNA (TERRA)[52,53]. The role of TERRA in HCC prognosis has been recently studied; it is downregulated in HCC and causes poor prognosis due to metastasis and cell growth, as studied *in vivo* and *in vitro*[54]. Through bioinformatics analyses, several regulatory protein motifs (regulating TERRA) at the end of chromosomes were identified and confirmed through experimental siRNA transcription on HeLa cells, when transfected[55]. Determination of the mechanisms of control of telomere homeostasis and telomerase enzyme will enable researchers to discover drugs that could modify this pathway, in order to cure cancer proliferation, and metastasis (Figure 3). I am involved in ongoing research in this area.

Other important molecular pathways

Other important molecular pathways that are studied in HCC are shown in Table 1. (1) Proliferation pathway is enhanced through the inhibition of various transcription factors (TFs). TFs present a form of differentiation therapy, which decreases cancer growth[56]. (2) Cellular growth in HCC: growth factor dysregulation causes disturbance in hepatocyte growth, and is considered as a treatment option for HCC[57]. (3) Angiogenesis in HCC: diagnosis and prognosis of HCC have different associations with various growth factors, including vascular endothelial growth factor (VEGF),

Table 1 Molecular pathways affected in hepatocellular carcinoma and their related protein-coding genes[14,33,34] and KEGG pathways database[35]

Function	Pathways and genetic regulators
Proliferation	Wnt pathway: MYC, FGF19, APC, AXINMAPK/ERK signaling pathway: mTOR, ERK 1/2
Cell growth and angiogenesis	RTK/RAS/PI(3)K pathway: PIK3CA, VEGF, EGF, MET, KRAS, PTEN, AKT1/2, FGFR1, NF1, TSC1/2, TGF-β pathway: SMAD2/3, SMAD4
Apoptosis	TP53 signaling pathway: MDM4, MDM2, CDKN2A, RPS6KA3
Cell immortality	Telomerase production: TERT
Cell cycle progression	RB1, CCND1, CDK4, CCNE1
Cell differentiation	HNF1A
Autophagy	RAS/RAF/MEK/ERK pathway, PI3K-AKT (AKT kinase)-mTOR pathway, and Wnt/β-catenin signaling pathway: Becilin-1, ATG3, ATG5, ATG7
Inflammatory response	IL-6 stimulation: STAT3, HNF1, IL6ST, GNAS
Chromatin modifiers	BAP1, ARID1A/B, IDH1/2, SMACA4, KMT2D

Wnt: Wingless and Int-1 (combined word); FGF19: Fibroblast growth factor 19 coding gene; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; mTOR: The mechanistic target of rapamycin; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; KRAS: K-Ras coding gene; PTEN: Phosphatase and tensin homolog coding gene; FGFR1: Fibroblast growth factor receptor 1 coding gene; SMAD family: Signal transducers for receptors of the transforming growth factor beta coding genes; Tp53: Tumor protein P53; MDM2: E3 ubiquitin ligase to degrade p53 coding gene; *RPS6KA3*: Ribosomal Protein S6 Kinase A3 coding gene; TERT: Telomerase reverse transcriptase coding gene; CCND1: Cyclin D1 Coding gene; CDK4: Cyclin-dependent kinase 4; CCNE1: Cyclin E1 coding gene; HNF1A: HNF1 Homeobox A coding gene; IL: Interleukin; ATG: Autophagy Related coding gene; *STAT3*: Signal transducer and activator of transcription 3; GNAS: Guanine nucleotide binding protein; BAP1: BRCA1 associated protein-1; ARID1A/B: AT-rich interactive domain-containing protein 1A/B; IDH1/2: Isocitrate dehydrogenase 1/2; KMT2D: Lysine Methyltransferase 2D coding gene.

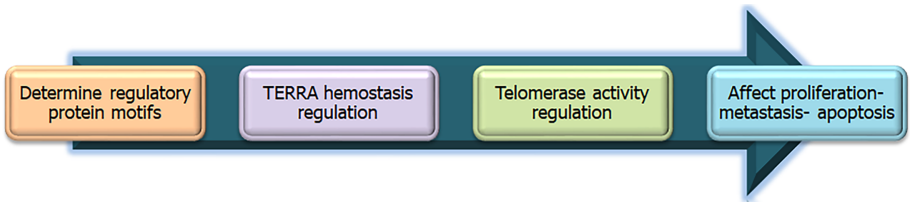

Figure 3 Proposed mechanism of drug development using bioinformatics and molecular knowledge about telomere homeostasis.

epidermal growth factor, transforming growth factor, *etc*. (4) Inflammatory response: for example, the effect of the interleukin pathway, and chronic inflammatory response in chronic hepatitis or steatohepatitis could result in activation of this pathway. And (5) Cell cycle progression: as mentioned earlier in control of cyclin D1, this could also form a suitable drug target.

COMPUTATIONAL METHODS USED IN MOLECULAR PATHWAY DISCOVERY

Interactive networks formed by data mining

Genetic networks formed through data mining are formed of two types: supervised learning, which mainly investigates data through statistical analysis of the patterns of coexpression presented in different genes; and unsupervised learning, which mainly deals with the discovery of genetic signatures to predict occurrence of certain diseases [58]. Both are considered methods of artificial intelligence and machine learning. Identifying the coexpression of genetic patterns for diagnosis and prognosis, through machine learning, could help formulate personalized therapeutic targets and advance precision medicine[59]. Figure 4 shows the pathways of bioinformatics analysis, and the general directions aimed in using *in silico* analysis.

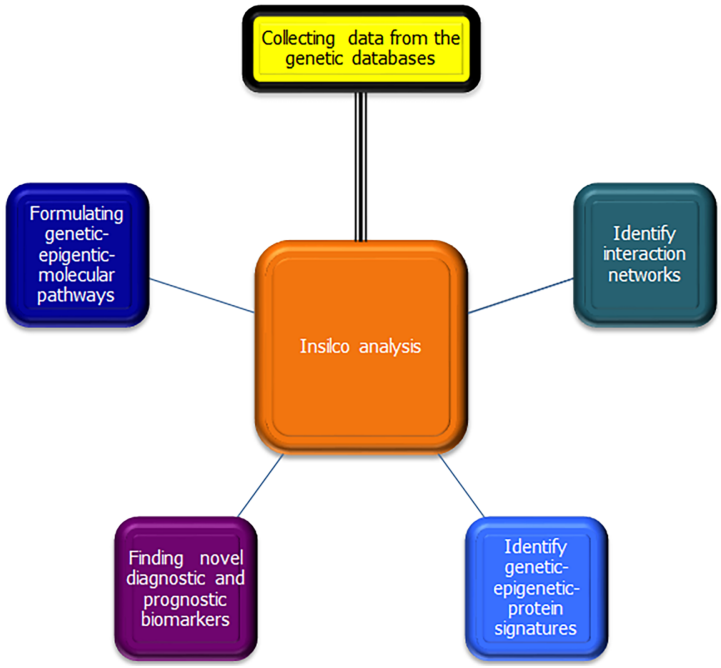

Figure 4 Pathways and aims of bioinformatics analysis.

Forming a miRNA signature

miRNA signature is a group of miRNAs that act collectively as one diagnostic or prognostic biomarker for a certain disease. By using the most relevant and lowest number of miRNAs to achieve the highest possible sensitivity and specificity of this biomarker in diagnosis or prognosis, we can create a relevant signature. Recently a group of scientists used support-vector-machine-based technology to assess the relation of miRNA signatures with clinical staging of HCC. The results showed 23 miRNAs with collective high sensitivity and specificity in differentiating early from late HCC, while seven miRNAs helped to determine the prognosis and survival in HCC patients[60].

Forming of prognostic biomarker coexpression signatures in HCC

Genetic or protein signatures formed by alignment of different sequences, preferably through multiple sequence alignment, could provide information about the "most conserved" sequence in a protein or gene or miRNA, through comparison between genes inherited by different species with a common ancestor (homologs), including similar genes in different species (orthologs), or different genes in the same species (paralogs). Coexpression signatures might help to categorize proteins or genes in different familial sets to predict their prognostic effect. There are different types of coexpression signatures including patterns, fingerprints and profiles[61-63].

A group of researchers collected different known HCC prognostic genes from various genetic and oncological databases, then through Lasso-Cox modeling a single prognostic signature, composed of the five genes *CCNB2*, *DYNC1LI1*, *KIF11*, *SPC25* and *KIF18A*, was tested in HCC tissues from patients by immunohistochemistry against HCC survival[64]. Another group of researchers found a single 14-gene signature for the prediction of HCC outcome[65].

A recent proteomic study used data mining to examine a new prognostic predictor protein signature. They found that four proteins, proliferating cell nuclear antigen, MutS homolog, cyclin-dependent kinase 1 and asparagine synthetase, were expressed in HCC tissue, and formed a single protein signature that predicted HCC survival. Most studies have used clinical proteomic databases including Clinical Proteomic Tumor Analysis Consortium (CPTAC) and Cancer Proteome Atlas (TCPA) as the source for genetic data collection[66].

Forming a gene coexpression network

A research group used 389 differentially expressed genes (retrieved from the GEO database) to build a gene coexpression network using the Robust Rank Aggregation method to aggregate ranked genes, and found that 40 hub genes (*i.e.*, functionally

significant in the module formed) were linked to HCC diagnosis, including 30 hub genes that were linked to HCC prognosis. Subsequent clinical validation of those most significant (only three novel biomarkers), was done on 32 HCC patients, showing upregulation in all three biomarkers, and their upregulation was associated with advanced tumor staging and worse prognosis. Those three novel biomarkers had not been assessed before in HCC, and all were linked to the regulation of cellular methylation process[67].

Bioinformatic analysis for HCC-therapy drug candidates (through molecular pathways or drug docking)

An important area in the discovery of novel drug candidates is drug-docking analysis. This is the first line of drug discovery in the era of bioinformatics, and has provided us with research on new applications of existing drugs and discovery of novel ones. This area, despite being interesting, is strictly used by pharmacology and biology specialists, and it is only during clinical validation that clinicians become aware of it during assessment of new drugs in clinical trials.

Following *in silico* validation, the first step is *in vitro* validation on hepatocellular culture, and later *in vivo* through animal or preclinical trials.

The first human trial is considered Phase 0 and is conducted only on healthy humans, as an exploratory phase prior to examining the treatment on affected patients. Later in Phase I/II, we establish primarily the safety and secondarily the efficacy, while Phase III concentrates mainly on establishing the efficacy of the drug. Finally, the post-marketing phase (Phase IV) determines the effectiveness of the drug in real life settings. Both Phase III and IV also ascertain the occurrence of adverse events (*i.e.*, safety) in real life settings[68]. In case of known and established drugs already in use for other illnesses, drugs discovered through molecular docking can bypass the animal trials and Phase 0, and go straight to phase I/II clinical trials (Figure 5).

In HCC, many studies considered molecular docking as a way to discover new drug targets. Different pharmacological compounds were considered as drug targets, for example, berberine, which affects the PI3K/AKT signaling pathway[69], or phytoconstituents of *Cocculus hirsutus* (coclaurine, haiderine and lirioresinol), which affects the VEGF receptor pathway[70]. A recent study used both molecular docking and genetic networks to design anti-HCC drugs from an ancient traditional Chinese medicine SiNiSan (SNS). SNS affects primarily the p53 pathway, thus regulating apoptosis[71].

Drug docking requires knowledge and experience in computational programs and algorithms. An easier and more approachable way to search for drug candidates is through selecting pharmacogenic compounds achieved by bioinformatic analysis of different molecular pathways, then proceed to *in vitro* analysis in cellular culture, followed by *in vivo* analysis in animal trials, then all through the aforementioned steps of validation. Our team has just published a paper on this topic, where cyan was used as an antioxidant for the inhibition of HCC proliferation, through modulation of the cell cycle in Wistar rats. The drug effect resulted in lower levels of expression of long noncoding RNA MALAT1, and tubulin 1 mRNA, and higher levels of expression of miR-125b. We chose this drug target through *a priori* bioinformatic analysis, followed by laboratory confirmation, and later by *in vivo* animal trials[72].

Other areas of bioinformatics research include whole-exome sequencing, transcriptome sequencing, and cistrome analysis[73].

LIMITATIONS TO USING *IN SILICO* ANALYSIS AND CLINICAL VALIDATION

Cost-effectiveness

Data mining is an option to examine the association between genetic material and clinical diseases. Meanwhile, the data collection cost is high. Moreover, most genetic and epigenetic biomarkers incur a high cost for laboratory assessment, mostly supplemented by grants or national or international funding.

Cost-effectiveness of applying those novel biomarkers for general clinical practice has yet to be determined. This needs large-scale population studies, and specifically designed cost-effective models[74-76].

Generally speaking, a novel biomarker should be cost-effective to be applicable in clinical practice guidelines after its wide-scale validation. Ultrasound has proven to be cost-effective in screening, with or without the addition of AFP, as a part of the two-stage biomarker–ultrasound screening[77]. This is a critical issue, not only in develo-

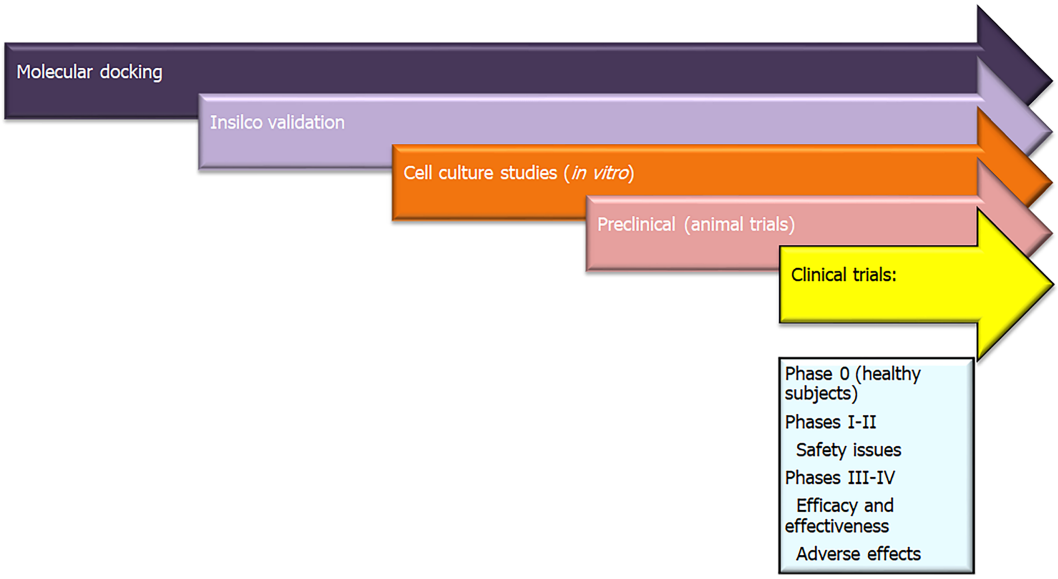

Figure 5 Role of molecular docking from drug discovery to clinical use.

ping and low-income countries but also in developed countries, while designing their clinical guidelines by healthcare system authorities.

Another problem faced is that laboratory analyses mostly require highly specialized researchers to handle the genetically fragile materials efficiently and without contamination or destruction. Preferably, this kind of research is conducted in highly specialized research laboratories for genetic analysis. Bioinformatics laboratories should always be constructed as a part of these physical laboratories.

CONCLUSION

The future holds out hope for personalized medicine, where we can treat HCC on an individual level, through assessing the genetic and epigenetic background of the patient, and then planning a specified management, considering the highest benefit and the lowest risk to the patient. The future also offers the promise of early detection of HCC, which has been the main obstacle in achieving our goal of cure, as most of the cases are diagnosed beyond the reach of curative methods, in late clinical stages.

Moreover, we can offer the chance for prognostic prediction of overall survival and tumor recurrence in HCC patients.

Proteomics, genomic, epigenomic and transcriptomic analyses provide massive data on the expression profiles of HCC; however, we are still unclear of their exact role or underlying mechanisms of action. Future studies are needed to integrate these data to provide a clear picture of the disease[66]. For example, S100A9 and granulin protein affect the progression of tumor and metastasis[78], and the inclusion complex of curcumin/β-cyclodextrin polymer prohibitsgrowth of HepG2 cell line[79]. These examples provide diagnostic and prognostic biomarkers for HCC severity and clinical progression, and further research on the affected molecular pathways as possible therapeutic targets specific to each patient, i.e., precision medicine[80,81].

Personalized medicine and individual planning for the management of patients with HCC are the future of medicine. To achieve this we need a multidisciplinary team of hepatologists, oncologists, clinical pharmacists, hepatic surgeons, interventional radiologists, nursing teams, psychiatrists, and social workers. All this should take place in specialized facilities, such as tertiary or specialized hospitals, which deal with these special types of cases. These facilities must include data storage access to a genetic bank, a blood and tissue bank, along with the required bioinformatics specialists to enter, retrieve and analyze data when needed. Finally, supportive teams of social workers, supporting family members and friends, while having effective communication with the medical team, are all essential in procuring the best possible outcome for the patient.

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REVIEW

Current treatment strategies and future perspectives for gastrointestinal stromal tumors

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Abstract

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that originate from the gastrointestinal tract, mostly from the stomach. GISTs are derived from the myenteric interstitial cells of Cajal and are caused by several mutations in the c-kit and platelet-derived growth factor receptor genes. Clinically, GISTs are detected by endoscopic and imaging findings and are diagnosed by immunostaining. Surgery is the first line of treatment, and if the tumor is relatively small, minimally invasive surgery such as laparoscopy is performed. In recent years, neoadjuvant therapy has been administered to patients with GISTs that are suspected of having a large size or infiltration to other organs. Postoperative adjuvant imatinib is the standard therapy for high-risk GISTs. It is important to assess the risk of recurrence after GIST resection. However, the effect of tyrosine kinase inhibitor use will vary by the mutation of c-kit genes and the site of mutation. Furthermore, information regarding gene mutation is indispensable when considering the treatment policy for recurrent GISTs. This article reviews the clinicopathological characteristics of GISTs along with the minimally invasive and multidisciplinary treatment options available for these tumors. The future perspectives for diagnostic and treatment approaches for these tumors have also been discussed.

Key Words: Gastrointestinal stromal tumor; Minimally invasive surgery; Laparoscopic surgery; Imatinib; Neoadjuvant therapy; Risk assessment

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Core Tip: Radical resection is the most effective treatment for gastrointestinal stromal tumors, but there are other options including minimally invasive surgery and multidisciplinary treatment, which involves the use of neoadjuvant therapy in consideration of tumor size and location. Combination with tyrosine kinase inhibitors is important for maximizing the therapeutic effect of surgery. To predict the effect, it is important to examine the presence of tumor mutations, including type, location of the mutation, and molecular subtype. We herein discuss the current treatment strategies for gastrointestinal stromal tumors and promising treatments based on clinical trials.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare tumors that account for 3% of all gastrointestinal tumors. GISTs originate from spindle-shaped cells known as Cajal cells, which behave as pacemakers and are normally found in the proximal muscles surrounding the intermuscular plexus of the gastrointestinal tract[1]. Hirota *et al*[2] reported that receptor tyrosine kinase KIT expression was observed in most GISTs; they also suggested that GISTs usually exhibit gain-of-function mutations in the c-kit gene encoding KIT and may be caused by a specific genetic abnormality[2].

The standard treatment for GISTs is radical resection; for tumors classified as high-risk, the standard treatment includes the administration of adjuvant imatinib for at least 3 years post-surgery[3]. This is because it is difficult to determine whether a GIST is benign or malignant even by pathological examination. For adjuvant therapy, the risk of GIST recurrence has been stratified by assessing the mitotic index, tumor size, and tumor location.

In addition, surgical approaches have been diversified according to the size and location of the tumor. Less invasive surgical procedures such as laparoscopy and laparoscopic and endoscopic cooperative surgery (LECS) have been performed for small GISTs, while preoperative chemotherapy is used to improve the probability of complete resection and prognosis for giant GISTs. Furthermore, when considering the selection of preoperative and postoperative drug treatment, genetic analyses have made it possible to predict, to some extent, the therapeutic effect, recurrence risk, and prognosis.

The purpose of this review is to provide an overview of the clinical features, its diverse treatment modalities, and strategies for genetically informed drug therapy of GISTs.

MANAGEMENT OF GIST

The National Comprehensive Cancer Network (NCCN) Guidelines Version 1.2021 was published on October 30, 2020, with the aim of describing basic treatment strategies for GIST[4]. If GISTs are suspected on endoscopy, imaging, and endoscopic ultrasound (EUS), then an EUS-guided puncture can be performed to confirm the diagnosis. An abdominal/pelvic contrast computed tomography (CT) or abdominal/pelvic contrast magnetic resonance imaging is recommended for every patient. In the case of submucosal tumors (SMTs) measuring less than 2 cm, the clinician should consider performing periodic endoscopic and radiographic surveillance. If there is a trend towards increase or high-risk features on EUS (unclear borders, cystic degeneration, ulceration, hemorrhage, and heterogeneity), curative surgery must be considered whenever possible[5]. For SMTs measuring over 2 cm, surgery is recommended if findings on imaging are suspicious of GIST, if there is a trend towards increase, or high-risk features. For SMTs measuring over 5 cm or if symptoms are observed (bleeding and pain, among others), surgery is recommended even if the diagnosis is

not confirmed.

When GIST is suspected, the treatment strategy differs depending on whether complete resection is possible. For resectable tumors with minimum morbidity, surgery is recommended; resection should be accomplished with histologically negative margins. For tumors that are not resectable without significant morbidity, administration of neoadjuvant therapy is the appropriate approach. In these cases, a biopsy is needed for confirming the diagnosis of GIST and for genetic examination. If the tumor is unresectable or if there is metastatic disease, tyrosine kinase inhibitor (TKI) treatment should be initiated.

SURGERY

For primary, non-metastatic GISTs, radical resection is the main treatment. Securing a margin at the time of excision is critical, as clean margins will affect the prognosis. For GISTs that have invaded or adhered to surrounding organs or viscera, *en bloc* resection including surrounding tissues is necessary to achieve R0 resection.

However, due to anatomical constraints, especially when the tumor is located in the esophagus, duodenum, and rectum, invasive surgery is often required; high complication rates are a problem. Conversely, when minimally invasive local resections are performed, surgical margins and long-term outcomes are questionable. Wei *et al*[6] retrospectively evaluated the outcomes of pancreaticoduodenectomy (PD) versus local resection in duodenum GISTs. The short time results were better in the local resection group, and there was no difference in prognosis based on the surgical procedure. They reported that tumor size and location were independent prognostic factors[6]. Therefore, for GISTs located in the mesenteric side of the second portion of the duodenum, PD is generally recommended; however, enucleation is recommended if the tumor is less than 5 cm in size. Wang *et al*[7] reported that in rectal GISTs, local excision provided a higher rate of anorectal preservation, shorter operative times, and fewer postoperative complications than radical resection, and that the long term results were similar in terms of recurrence-free survival (RFS). Based on these results, local resection and minimally invasive surgery are recommended whenever possible for GISTs that occur in anatomically complex regions.

Since GISTs rarely metastasize to the lymph nodes, routine lymphadenectomy is not necessary unless the lymph nodes are enlarged. However, caution is required in the case of wild-type GISTs. Most GISTs that occur in adults are caused by mutations in the *KIT* or platelet derived growth factor receptor (*PDGFRA*) genes, but 10%-15% of GISTs in adults and 85% in children are wild-type GISTs. Wild-type GISTs primarily affect young females; the main site is generally gastric, and they are multifocal yet indolent[8]. The pathogenic mechanism of wild-type GISTs is unknown, but one possible cause is dysfunction of the succinate dehydrogenase (SDH) complex in tumor cells. Along with paragangliomas, this type of GIST, is a component of the Carney-Stratakis syndrome, characterized by germline mutations of the SDH subunit[9]. Wild-type GISTs are also associated with pediatric GISTs and non-familial tumors; this is known as the Carney triad (wild-type GISTs, paraganglioma, and pulmonary chondroma) that is not associated with *SDH* germline mutations[10]. In SDH-mutant GISTs, lymph node metastases are frequently observed. Boikos *et al*[11] reported that in SDH-mutant GISTs, the incidence of nodal lesions was as high as 65%; half of them had lymph node metastasis. Therefore, resection of enlarged lymph nodes should be considered in patients with SDH-mutant GIST.

Resectable GISTs with minimal morbidity

Laparoscopic and LECS: Laparoscopic surgery is considered for selected GISTs of small size located in easily accessible locations. Especially for tumors less than 5 cm, laparoscopic resection is acceptable[12]. In a systemic review and meta-analysis, laparoscopic surgery was recognized to be safe and feasible due to less intraoperative blood loss, early postoperative recovery, shortened hospital stay, and a lower rate of postoperative complications[13]. However, when performing laparoscopic resection, it is essential to obtain negative resection margins for complete resection of the localized tumor; in addition, great care should be taken to avoid capsule damage to prevent tumor spillage[14].

When the tumor is located near the cardia, partial gastrectomy should be considered instead of proximal gastrectomy. However, if the tumor is of luminal-growth type and close to the cardia, an extensive resection of the margins is often required. Minimum resection margins can be challenging and will often result in a proximal gastrectomy.

In such cases, the lesion can be resected to the minimum necessary extent by observing the tumor from the lumen with an endoscope and determining the excision line. Hiki *et al*[15] first established a technique for performing minimally invasive local excision using a laparoscope and an endoscope; this was the first report on LECS in 2008. Since then, many facilities have introduced LECS in Japan, and evidence on its usefulness has been reported. A method based on a similar concept attracted attention in the 2000s; it involved completion of endoscopic treatment with laparoscopic assistance as part of the Natural Orifice Transluminal Endoscopic Surgery (NOTES) and was reported as hybrid NOTES[16]. Notably, intraoperative endoscopy is becoming increasingly popular for laparoscopic GIST resection, especially when the tumor is less than 3 cm or the location is difficult to access[17]. In Japan, gastric GISTs are often found to be relatively small; many LECS procedures have therefore been performed. To date, five representative LECS techniques have been developed.

Classical LECS is an extremely efficient method, because each step is simple and clear, technically easy, and surgery can be completed in a relatively short time. In addition, since the lesion is collected *via* the abdominal wall, there is no restriction on the size of the tumor; this is one of the merits of this procedure. However, this procedure requires opening of the stomach wall; there is therefore a potential risk of leakage of gastric contents or tumor into the abdominal cavity. Thus, this procedure should be applied with caution in tumors where the mucosal surface is exposed, such as in SMT with ulcers. In such cases, the non-open technique described below should be selected.

Inverted LECS[18] is a technique that prevents the contents of the stomach from leaking into the abdominal cavity. The edge of the resected gastric wall is first stitched and lifted, and the tumor is inverted into the stomach cavity. After the tumor is dropped into the stomach and removed orally using an endoscope, the stomach dissection line is temporarily closed by hand suturing and completely closed with stapling. Inverted LECS can prevent gastric juice from leaking to some extent, but it may not be applicable for all sites such as posterior wall lesions, among others, as it is not an entirely non-open technique. Therefore, completely non-open techniques were developed, such as non-exposed endoscopic wall-inversion surgery (NEWS)[19-21], closed-LECS[22], and a combination of laparoscopic and endoscopic approaches to neoplasia with a non-exposure technique (CLEAN-NET)[23,24].

NEWS was first devised as a way to resect early gastric cancer without opening the stomach wall[19]. The first step is to place an incision in the seromuscular layer around the tumor using a laparoscope; after pushing the tumor into the luminal side of the stomach, the seromuscular layer is continuously sutured. The next step involves making an endoscopic incision in the submucosa surrounding the intruded tumor. The lesion is then dissected and retrieved orally. The advantage of this technique is that the incision can be made under direct visual observation with an endoscope or laparoscope, while the tumor resection is completely closed.

Kikuchi *et al*[22] reported on a similar closed LECS technique. After local injection of the submucosal layer, a mucosal incision is made with an endoscope; this is followed by suturing of the serosal muscular layer while inverting the lesion with a spacer. The seromuscular layer is incised again *via* an endoscope. The tumor is then retrieved orally, and the mucosal edge is closed using the same procedure as in NEWS. These procedures are excellent, especially for intraluminal GISTs; this is because they allow for an appropriate resection line. These techniques are very useful for small GISTs. However, one limitation is that the diameter of the tumor can only be up to 3 cm, because the resected tumor needs to be removed orally.

CLEAN-NET was developed by Inoue *et al*[23]; it is a non-exposed excision technique that involves incision of the serosa and muscularis, while preserving the continuity of the mucosa[23]. Unlike a normal laparoscopic local resection, this procedure allows for minimal local excision by first incising the serosa and muscularis, stretching the mucosa, and then pulling the lesion outward. The tumor is collected trans-abdominally, allowing for a relatively large GIST of up to 5 cm to be retrieved. However, this method tends to provide a slightly larger margin, because all sections are performed from the abdominal cavity. It is therefore not suitable for areas where a large surgical margin cannot be obtained, such as near the cardia.

The features of each LECS are summarized in Table 1. The choice of each technique depends on the size, location, and growth pattern of GISTs. Especially for ulcerated GISTs, the non-open techniques of NEWS, closed LECS, and CLEAN-NET are good options. In addition, NEWS and closed LECS are good alternatives for intraluminal type GIST and closed LECS for the extraluminal type[25,26].

Table 1 Various laparoscopic and endoscopic cooperative surgery procedures for gastrointestinal stromal tumors

Procedure	Yr	Author	Indication	Non-exposure	First approach	Preferred type and location	Extraction site	Suturing
Classical LECS	2008	Hiki	< 5 cm ulcer (-)	No	Endoscopic	Intraluminal > extraluminal; Anterior wall	Trans abdominal	Hand or mechanical
Inverted LECS	2012	Nunobe	< 5 cm ulcer (±)	No	Endoscopic	Intraluminal > extraluminal; Anterior wall	Either site	Hand or mechanical
Closed-LECS	2017	Kikuchi	< 3 cm ulcer (+)	Yes[1]	Endoscopic	Intraluminal < extraluminal; Anterior wall	Trans oral	Hand
NEWS	2011	Goto	< 3 cm ulcer (+)	Yes	Laparoscopic	Intraluminal < extraluminal; Anterior wall	Trans oral	Hand
CLEAN-NET	2012	Inoue	< 3 cm ulcer (+)	Yes	Laparoscopic	Intraluminal < extraluminal; Anterior wall	Trans abdominal	Mechanical
PEIGS	1995	Ohashi	< 3 cm ulcer (+)	No	Laparoscopic	Intraluminal > extraluminal; Posterior wall	Either site	Hand or mechanical

[1]Open the gastric wall.

LECS: Laparoscopic endoscopic cooperative surgery; NEWS: Non-exposed endoscopic wall-inversion surgery; CLEAN-NET: Combination of laparoscopic and endoscopic approaches to neoplasia with a non-exposure technique; PEIGS: Percutaneous endoscopic intragastric surgery.

Percutaneous endoscopic intragastric surgery: The percutaneous endoscopic intragastric surgery (PEIGS) technique was first reported by Ohashi *et al*[27]. A method using three indwelling intragastric ports had been devised; since then, intragastric surgery by various methods such as single incision and needlescopic PEIGS has been reported [25]. PEIGS is a surgical procedure in which an endoscope and forceps are inserted into the stomach lumen through the abdominal and anterior gastric walls. This procedure is useful for intraluminal gastric SMT. In this case, determining an adequate resection margin is not easy because of the difficulty in confirming the tumor location from outside the gastric wall. Especially for lesions on the posterior wall of the cardia, the laparoscopic approach is complicated and relatively time-consuming. In contrast, intragastric surgery can obtain an easy approach and good operative view; PEIGS is therefore suitable for such cases. The problem with this procedure is the risk of surgical site infection secondary to pseudo-perforation. However, Kanehira *et al*[28] reported the incidence of surgical site infection to be approximately 2%, which was well within the acceptable range.

Endoscopic resection: There are various reports on the removal of intraluminal SMTs with an endoscope alone[29-31]. In these procedures, endoscopic full-thickness resection may be performed for intraluminal SMT originating in the muscularis propria (MP) layer. This procedure involves incising the MP layer around the SMT first; the serosal layer is then incised to generate perforation. The SMT with surrounding tissue is then removed using a snare, and the perforated gastric wall is closed using an endoscopic clip and an endloop[31]. However, this procedure involves the risk of leakage of the gastric contents due to pseudo perforation. To solve this problem, over-the-scope-clip and snaring are being developed as a full-layer suture device[32]. In this procedure, the over-the-scope-clip is first placed in the lesion, and the base of the lesion is completely resected by the snare to prevent pseudo-perforation. This technique is especially useful for small SMTs of 2 cm or less.

Newer therapies, such as endoscopic ultrasound alcohol ablation, have shown promising results. EUS-guided injection of 1.5 mL of 95% ethanol was performed for primary or metastatic GISTs without technical incidents[33]. While long-term follow-up is required to ascertain its efficacy and safety, it may be considered for high-risk patients.

Resectable GISTs with significant morbidity

Neoadjuvant therapy: Surgical resection is the mainstream for GIST treatment, and

complete resection without damage caused by pseudo-capsule resection is essential. If the tumor is large and is suspected to have infiltrated to other organs, the complete resection rate may decrease and the recurrence rate due to intraoperative tumor rupture may be higher. Additionally, even if complete resection of a larger tumor is achieved, the risk of recurrence increases with tumor size[34]. For such cases, the rate of extensive surgery is increased; this is associated with significant morbidity. Preoperative treatment with imatinib is therefore attempted in such cases, as tumor shrinkage is essential for ensuring a negative surgical margin and avoiding the risk of rupture from subsequent surgical procedures.

Function-preserving surgery is another aim of preoperative administration of imatinib. When considering function preservation by avoiding extended surgery, the effect of neoadjuvant therapy is greatly influenced by the location of the tumor. Tumor shrinkage at the esophagogastric junction can convert a total gastrectomy into a local resection. In duodenal GISTs close to the pancreatic head, PD may be avoided by neoadjuvant therapy. Neoadjuvant imatinib allows for preservation of the anal sphincter in certain rectal GISTs. Indeed, neoadjuvant imatinib has been commonly administered in retrospective series for GISTs located in such locations.

Based on two large-scale clinical databases, the BFR14 trial[35] and the European Organization for Research and Treatment of Cancer (EORTC) Soft Tissue and Bone Sarcoma Group[36] from four Dutch institutions[37], several studies have reported on neoadjuvant imatinib for GISTs. Tielen *et al*[37] performed a cohort study on preoperative imatinib for locally advanced GISTs. All tumors were over 5 cm or ill-located for surgery. The response rate (RR) to preoperative treatment was 83%, and the R0 resection rate was 84%, with no tumor perforation occurring during the operation. The 5-year progression-free survival (PFS) and overall survival (OS) were 77% and 88%, respectively. The PFS tended to be better in the neoadjuvant imatinib group, but statistical significance was not detected. Among reports on neoadjuvant imatinib, the EORTG Soft Tissue and Bone Sarcoma Group study is the largest; the results of preoperative administration of imatinib at a dose of 400 mg for locally advanced GISTs have been reported. The average duration of imatinib administration was 40 wk. In this report, the RR was 80%, and the R0 resection rate was 83%. Five-year disease-free survival and disease-specific survival were 65% and 95%, respectively. The postoperative complication rate was 15%, although surgical re-intervention was required in only 3%. The authors concluded that preoperative imatinib administration appears safe, and it is a promising treatment for patients with locally advanced or marginally resectable primary GISTs.

The contribution of preoperative imatinib therapy varies depending on the location of the tumor and is considered particularly effective in the esophagogastric junction, duodenum, and rectum. Jakob *et al* showed that those who received neoadjuvant imatinib for rectal GISTs had a significantly higher rate of negative surgical margins than those who did not receive treatment. All patients with positive resection margins and postoperative recurrence had not received preoperative treatment. In patients undergoing preoperative imatinib therapy for locally advanced rectal GISTs, a complete resection rate was obtained in 77%, which is higher than that of patients not treated preoperatively[38]. These results suggest that preoperative imatinib was associated with an increased R0 resection rate and also allowed for surgery in anatomically difficult areas.

Three prospective multicenter phase II trials have evaluated the efficacy of neoadjuvant imatinib in locally advanced GISTs[39-42]. RTOG 0132 was the first trial and reported short- and long-term results. Eligible patients had GIST with primary disease greater than 5 cm or metastatic/recurrent disease greater than 2 cm. Thirty-one of the 53 patients had primary GIST and were evaluated as the preoperative imatinib group. Preoperative imatinib was administered at a dose of 600 mg for 8–12 wk until surgery, and postoperative adjuvant therapy was planned for 2 years. In this report, the RR was 7%, and R0 resection rate was 68%. The lower RR compared to other reports was attributed to the shorter duration of neoadjuvant imatinib therapy. The 5-year PFS and OS were 57% and 77%, respectively. This trial has proved to be feasible and was not associated with significant postoperative complications.

The results of a phase II trial on preoperative imatinib therapy for large gastric GISTs in Japan and South Korea have been reported recently. For patients with large gastric GIST (> 10 cm), imatinib was administered at a dose of 400 mg for 6-9 mo until surgery. The primary endpoint was the R0 resection rate, and the secondary end points were RR, PFS, and OS. The RR was 62%, and R0 resection rate was 91%. At a median follow-up of 32 mo, the 2-year PFS was 89% and OS was 98%. These results suggest that neoadjuvant imatinib administered at a dose of 400 mg for 6-9 mo would be a promising treatment for patients with high-risk GISTs. Long-term follow-up is

expected to prove the contribution of neoadjuvant imatinib to survival in high-risk GISTs.

These advanced treatments are expected to improve the prognosis, and many studies have reported such results (Table 2). Neoadjuvant therapy is expected to preserve organ function, avoid tumor rupture, reduce complications, and ultimately prolong overall survival; however, the evidence of efficacy remains to be established.

Important aspects for neoadjuvant therapy: The NCCN and European Society for Medical Oncology guidelines recommend that GIST must be diagnosed pathologically if neoadjuvant therapy is to be considered[4,43]. Tissue sampling can be obtained by endoscopic or bowling biopsy, but sometimes this is not sufficient for confirming the diagnosis. Percutaneous biopsy and tissue collection by laparotomy are contraindicated due to the risk of peritoneal dissemination. However, Eriksson *et al*[44] reported that percutaneous biopsy of GISTs collects sufficient tissue and does not increase the risk of recurrence in patients who receive imatinib preoperatively[44]. In addition to GIST diagnosis, it is recommended to check for genetic mutations before starting preoperative treatment to ascertain whether the treatment is likely to be effective. KIT exon 11 and 9 mutants will respond to imatinib, but higher doses of imatinib are required for response in cases of KIT 9 mutations[45].

Nilotinib is a selective TKI with a potency similar to that of imatinib[46]. A randomized phase III trial on the efficacy and safety of nilotinib as a first-line treatment was conducted[47]. In this study, the PFS was higher with imatinib in the KIT exon 9 group but similar in the KIT 11 group. Thus, for patients with KIT exon 11 mutations who cannot receive imatinib, nilotinib is a promising preoperative agent.

It is also known imatinib has no therapeutic effect on GIST with the PDGFRA exon 18 D842V mutation, which has a poor prognosis. The NAVIGATOR study was a phase I trial to assess the efficacy and safety of avapritinib administration for unresectable GISTs patients, who tested positive for the PDGFRA exon 18 D842 V mutation[48]. In patients with PDGFRA exon 18 D842 V-mutant GIST, 88% had a response; 9% had complete responses, and 79% had partial responses. Based on the results of this trial, the Food and Drug Administration approved the use of avapritinib in adult patients with unresectable or metastatic GIST who have PDGFRA exon 18 mutations, including D842V mutations. Therefore, in patients with resectable GISTs associated with significant morbidity, and those having PDGFRA exon 18 mutations including the D842 mutation, neoadjuvant avapritinib is considered.

Evaluation of the response and treatment period: CT is the most used imaging modality to determine the effect of neoadjuvant imatinib; however, depending on the conditions, magnetic resonance imaging may be more useful for patients who are allergic to CT contrast media, those who have tumors located at specific sites such as the rectum, or those who require evaluation for liver metastases. CT can assess the change in both, tumor size and tumor viability. If imatinib has a therapeutic effect, the inside of the tumor is necrotic and degenerative, although the tumor size does not change at first. Evaluating metabolic rather than morphologic changes may therefore be more reliable for early treatment assessment. Therapeutic effect determination by the Response Evaluation Criteria In Solid Tumor criteria may also underestimate the response. The Choi Criteria[49], however, evaluates the size of the tumor and its density; it is therefore useful for evaluating the therapeutic effect of TKI. However, in order to measure changes in vascularization and to measure tumor density, CT should be obtained in arterial and portal phases[50]. Positron emission tomography (PET)/CT is highly sensitive for GISTs and can evaluate the effect of treatment earlier than tumor size changes. Previous studies have shown that PET/CT can predict imatinib response within 1-8 d[51]. Therefore international guidelines recommend early evaluation of response by PET/CT (within 2-4 wk) when neoadjuvant treatment with imatinib is administered, and rapid readout of activity is necessary[4].

The optimal duration of preoperative administration of imatinib is still unclear, but the most suitable timing for maximum effect is before secondary resistance is acquired. The pharmacological effect of imatinib is rapid, but this drug acts as a cytostatic agent; tumor shrinkage therefore takes time. In unresectable GISTs it takes an average of 3 mo for the tumor to shrink with imatinib; a plateau is reached at 6 mo[52]. In a study on patients with metastatic or unresectable GISTs, the median time to tumor progression was 12 mo; tumor progression occurred in half of the patients within 2 years of starting imatinib[53]. Tirumani *et al*[54] reported that in a cohort receiving neoadjuvant with imatinib, best response was achieved at wk 28; thereafter, a plateau response continued until wk 34[54]. These results suggest that the appropriate duration of preoperative imatinib may be for 6-9 mo.

Table 2 Studies on neoadjuvant imatinib therapy for gastrointestinal stromal tumors

Ref.	Clinical trial	Yr	Design	Endpoint	Cases	Agent/Dose	Patients	Duration	RR	R0 rate	Adjuvant imatinib	PFS	OS
Prospective study													
Eisenberg *et al* [39]	RTOG0132 trial	2009	Phase II	RFS	30 (all; 52)	Imatinib/600 mg	GIST (> 5 cm)	8-12 wk	7%	77%	24 mo	2-yr PFS; 83%	2-yr OS; 93%
Wang *et al*[40]	RTOG0132 (long follow up)	2012			31 (all; 53)							5-yr PFS; 57%	5-yr OS; 77%
Doyon *et al*[41]		2012	Phase II	RR	14	Imatinib/400 mg	Locally advanced GIST	6 mo	43%	79%	12 mo	4-yr DFS; 64%	4-yr OS; 100%
Kurokawa *et al* [42]	Asia	2017	Phase II	PFS	53	Imatinib/400 mg	Gastric GIST (> 10 cm)	6-9 mo	62%	91%	36 mo	2-yr PFS; 89%	2-yr OS; 98%
Retrospective study													
Blesius *et al*[35]	BFR14 trial	2011	Subset phase III	-	25	Imatinib/400 mg	Locally advanced GIST	4.2 mo (median)	60%	32%	13-24 mo	3-yr PFS; 67%	3-yr OS; 89%
Rutkowski *et al* [36]	EORTC	2012	Database	-	161	Imatinib/400 mg	Locally advanced GIST	40 wk (median)	80%	83%	At least 1 yr (56%)	5-yr DFS; 65%	5-yr DSS; 95%
Tielen *et al*[37]		2013	Database	PFS/OS	57	Imatinib/400 mg	GIST (> 5 cm) and/or ill-located for surgery	8 mo (median)	83%	84%	1, 2 yr or lifelong (58%)	5-yr PFS; 77%	5-yr OS; 88%

RFS: Relapse-free survival; RR: Response rate; PFS: Progression-free survival; OS: Overall survival; DSS: Disease-specific survival.

Postoperative therapy: In GIST classified as high-risk after curative surgery, adjuvant imatinib therapy is standard treatment; the recommended period of therapy is at least 3 years[55]. However, there is no consensus on postoperative adjuvant therapy for patients treated with neoadjuvant imatinib. In the RTOG0132 trial; the 5-year disease-free survival in patients who received adjuvant imatinib was better than that in patients who did not receive the drug. However, recurrence occurred within 2 years of completion of adjuvant imatinib. Therefore, for patients treated with neoadjuvant imatinib, postoperative adjuvant therapy is required for 3 years, similar to the period of adjuvant therapy required for high-risk GIST.

Surgical intervention for metastatic GIST

The treatment of unresectable, advanced, and recurrent GISTs is mainly based on TKI administration; however, surgical intervention may be possible in some cases. If the response to imatinib is good and the disease is controlled, surgery may be indicated. This includes cases of initially unresectable GIST that has responded well to imatinib

and become resectable, locally progressed GIST due to secondary resistance, low-volume stage IV disease, or cases requiring palliative surgery for symptoms such as bleeding or obstruction. If complete resection can be achieved, surgical intervention in combination with imatinib is more effective[56,57]. A retrospective study reported that GIST patients who respond to imatinib therapy have significantly higher complete resection rates and better PFS and OS than those who do not respond to imatinib. Additionally, two randomized controlled trials evaluated the efficacy of multidisciplinary treatment combining imatinib with surgical intervention for recurrent or metastatic GISTs[56,58]. Xia *et al*[56] investigated the efficacy of surgery and pre-and post-operative imatinib administration for advanced GISTs with liver metastasis and reported that the OS was better with surgical intervention. Furthermore, surgical resection offered better OS in GIST patients who had a poor response to imatinib therapy in the 6 mo prior to surgery. These findings suggest that in some cases, patients with liver metastases from GIST may have a better prognosis with surgical intervention than with imatinib alone. However, the indication for and optimal timing of surgery are still unclear, and future consideration is awaited.

Surgery after second line treatment such as sunitinib is considerably rare; however, certain retrospective studies report on its efficacy. Yeh *et al*[59] reported on the benefit of surgical intervention in metastatic GIST with local progression while receiving sunitinib. They reported fewer complications (15.3%) and significantly prolonged PFS and OS. Surgery may contribute to the suppression of events such as bleeding and ileus caused by the growth of tumors that have acquired secondary resistance to sunitinib; it may also improve disease control by removing resistant lesions. The results of cytoreductive surgery for GIST with local progression during regorafenib treatment in the third line have also been reported[60]. Although there is a bias in the retrospective study, the PFS and OS were 12.9 mo and 32.2 mo, respectively; these were better than those of patients who did not undergo cytoreductive surgery. However, it is important to note that the complication rate was as high as 33%, although the surgery was performed on relatively young patients with good performance status.

Based on the above findings, cytoreductive surgery for selected GISTs that have acquired resistance in the second and third line may provide local control, serve as a bridge to drug therapy, and ultimately improve prognosis.

RISK ASSESSMENT AND ADJUVANT THERAPY

The tumor size and mitotic index are important in assessing the risk of recurrence of GISTs, but it is difficult to assess whether a tumor is a benign or malignant based on these features alone. Miettinen *et al*[61] reported that in GISTs with a diameter of more than 10 cm and a mitotic index of ≤ 5 mitoses/50 high power field, the recurrence rate of small intestinal GIST is considerably higher than that of gastric GIST[61]. Therefore, in addition to tumor size and mitotic index, tumor site is also included in their classification. The Joenssu classification, that includes tumor location and considers tumor capsule rupture cases where recurrence is almost inevitable, is useful in that it efficiently selects only the group at high risk of recurrence[62].

As described previously, tumor size, mitotic count, and primary location are important in assessing the risk of GIST recurrence; however, measuring mitotic count on a slide is highly individualized and depends on the ability to distinguish the cells from other cells such as apoptotic bodies and pyknotic cells, among others. In SDH-deficient GISTs, mitotic count does not predict tumor behavior[63]. Therefore, at the basic research level, an attempt has been made to predict the risk of GIST recurrence by measuring gene expression related to DNA methylation; this has been shown to be an effective predictor[64].

Imatinib is administered as adjuvant therapy for the high-risk group after surgery, as GISTs generally harbor an imatinib-sensitive mutation. The most frequent *KIT* exon 11 mutations are sensitive to imatinib, whereas the PDGFRA exon 18 D842 V-mutation is considered to be imatinib-resistant. Tumor mutation analysis is important for estimating the therapeutic effect of imatinib; however, whether evaluation of tumor mutations is more useful than the above risk assessment is controversial. Under the circumstances, a study examined the indications for adjuvant therapy by gene mutation analysis. In GIST patients with *PDGFRA* mutations and *KIT* exon 11 duplication, mutation, or deletion of one codon, good RFS has been achieved with surgery alone. Therefore, this type of genetic variation is an independent factor that affects RFS beyond recurrence risk classification numbers. These results suggest that

adjuvant therapy is not necessary for these genetic mutations.

Three randomized phase III trials have reported on the efficacy of postoperative adjuvant imatinib (Table 3). The first trial was the ACOSOG Z9001 study by the American College of Surgeons Oncology Group. The major eligibility criterion was complete resection of the primary GIST, tumor diameter more than 3 cm, and positivity for KIT on immunostaining. In this study, imatinib administration for 1 year conferred significantly better RFS than placebo [98% *vs* 83%, hazard ratio (HR) = 0.35, $P < 0.0001$]. In the largest phase III trial, EORTC 62024 patients were randomly assigned to receive imatinib at a dose of 400 mg for 2 years or surgery alone. The high or intermediate-risk group with R0 or R1 surgical margins was eligible for inclusion. The 5-year imatinib failure-free survival was 87% in the imatinib administered group and 84% in the control group (HR = 0.79, $P = 0.21$); the primary endpoint was therefore not significant. However, when the high-risk subgroup was analyzed, there was a trend towards better imatinib failure-free survival in the imatinib group (79% *vs* 73%, $P = 0.087$). The results of these studies revealed that adjuvant imatinib improved RFS when administered to patients with operable GIST; however, its influence on OS remains uncertain.

In the open-label, multicenter, randomized phase III SSGXVIII/AIO trial, patients with GIST who underwent radical surgery but were at high-risk were enrolled; they received adjuvant imatinib therapy for 1 or 3 years after surgery. The primary endpoint was RFS; the secondary endpoints were OS and safety. The 5-year and 10-year RFS were 71.4% and 52.5%, respectively, in the 3-year group, and 53.0% and 41.8% in the 1-year group (HR = 0.66, $P = 0.003$). The 5-year and 10-year OS rates for the 3-year group were 92.0% and 79.0%, respectively; for the 1-year group, they were 85.5% and 65.3%, respectively. The difference was statistically significant (HR = 0.55, $P = 0.004$). Therefore, administration of adjuvant imatinib for at least three years is the standard treatment for patients in the high-risk group[3,55]. The cited article reported that approximately 50% of deaths may be avoided during the first 10 years after surgery with longer adjuvant imatinib treatment.

A study on long-term administration (5 years or more) has been reported in the phase II PERSIST trial[65]. The 5-year RFS was 90%, while the OS rate was 95%. Six of 7 patients who developed recurrence did so after completing adjuvant imatinib. Furthermore, among the patients with an imatinib-sensitive KIT exon 11 mutations, only 1 experienced recurrence, which occurred after imatinib was discontinued. This indicates that long-term imatinib administration in patients with imatinib-sensitive mutations is effective in preventing the recurrence of GIST. Two randomized trials on the effects of long-term adjuvant imatinib therapy, namely, sSGXXII and IMADGIST, are ongoing and their results are awaited.

SYSTEMIC THERAPY

Gene analysis

KIT mutations: Imatinib is expected to be more than 80% effective in patients with unresectable, advanced, and recurrent GIST; the median OS after treatment with imatinib is 50 mo[66]. However, the therapeutic effect depends on the sensitivity of the GIST to imatinib; this can be predicted to some extent by identifying gene mutations. The most frequent gene mutation is that of KIT exon 11 (65%), followed by that of exon 9 (10%). Approximately 90% of KIT exon 11 and 50% of KIT exon 9 mutation GISTs respond to imatinib; however, the therapeutic effect is different to a certain extent. In the EORTC study, GISTs with exon 11 mutations showed high efficiency to imatinib and increased PFS and OS than those with exon 9 mutations. However, the relationship between imatinib doses and therapeutic effects also differs by gene mutations. In GIST patients with KIT exon 9 mutations, increasing the dose of imatinib (800 mg/d) prolonged PFS. Conversely, in patients with KIT exon 11 mutations or wild-type GIST, imatinib dose escalation did not improve PFS. However, the contribution of imatinib dose increase to OS in exon 9 mutation cases was not clear even on meta-analysis; the finding has therefore remained controversial[45].

Mutations in exon 13 and 17 are very rare; compared to other mutations, they occur more frequently in the small intestine. Genetic mutations in secondary resistant GISTs are often found in exons 13 and 17; secondary mutations occur mostly in exon 13, which constitutes the adenosine triphosphate (ATP) binding domain, and in exon 17, which constitutes the activation loop[67]. Many secondary mutations in the ATP binding domain are sensitive to sunitinib even after imatinib resistance; however, most of the secondary mutations in the activation loop are resistant to both, imatinib and

Table 3 Clinical studies on adjuvant imatinib

Trial	ACOSOG Z9001	SSG XVIII/AIO	EORTC 62024	PERSIST-5
Study/yr	Phase III/2009	Phase III/2012, 2020	Phase III/2015	Phase II/2018
Number	359 (total: 713)	397 (199 *vs* 198)	454 (total: 908)	91
Eligible criteria	Tumor size ≥ 3 cm	High risk group	Intermediate and high-risk group	Intermediate and high-risk group
Treatment dose	400 mg/d	400 mg/d	400 mg/d	400 mg/d
Duration	1 yr *vs* placebo	1 yr *vs* 3 yr	2 yr *vs* placebo	5 yr
Risk classification				
High risk	NA	178 (89%)	266 (58.6%)	67 (74%)
Intermediate risk		15 (8%)	186 (41%)	24 (26%)
Etc.		6 (3%)	2 (0.4%)	
Residual tumor				
R0	325 (90.5%)	169 (85%)	381 (83.9%)	90 (99%)
R1,2	34 (9.5%)	30 (15%)	73 (16.1%)	0 (0%) 1; unknown
Tumor rupture				
No	NA	164 (82%)	404 (89%)	NA
Yes		35 (18%)	50 (11%)	
End point				
Primary endpoint	RFS	RFS	IFFS	RFS
Secondary endpoint		OS, safety	RFS, OS, safety	OS
Results	1-yr RFS; 98% *vs* 83% (HR = 0.35, $P < 0.0001$); OS: Not significant	5-yr RFS; 71% *vs* 53% (HR = 0.66, $P = 0.003$); 5-yr OS; 92% *vs* 86%; 10-yr OS; 79% *vs* 65%	5-yr IFFS; 87% *vs* 84% (HR = 0.79, $P = 0.21$); 3-yr RFS; 84% *vs* 66%; 5-yr RFS; 69% *vs* 63%	5-yr RFS; 90%; 5-yr OS; 95%; 45 (49%) pts early discontinuation of imatinib

NA: Not associated; RFS: Relapse-free survival; OS: Overall survival; IFFS: Imatinib failure-free survival.

sunitinib.

Mutations in exon 8 are even rarer, with only a few cases reported in the past and an estimated frequency of approximately 0.3%. The most common genotype of exon 8 mutations is Del-Asp419; the others known are ThrTyrAsp (417-419) Tyr. In pediatric mastocytosis, the reported type of c-kit mutation in exon 8 is Del-Asp419. Hartmann *et al*[68] reported that GIST patients with Del-Asp419 mutations had mastocytosis as well as multiple GISTs, suggesting an association. The most common sites are the small intestine and duodenum, and it appears to arise from extragastric sites. Many GISTs with exon 8 mutations have metastases at the time of diagnosis or are classified in the high risk group; this indicates the possibility of aggressive behavior. Sensitivity to imatinib has been demonstrated *in vitro*. In clinical practice, it has been administered as adjuvant therapy to the intermediate to high-risk group, with no observed recurrence for 24 mo[69].

PDGFRA mutations: Mutations in the PDGFRA gene account for 5%-10% of all GISTs and are found mostly in the stomach. Mutations are present in exons 12, 14, and 18, with mutations in exon 18 being the most common; the most common genotype was D842V. D842V mutations are resistant to imatinib, but sensitive to avapritinib. In D842V mutant GISTs, avapritinib was found to be highly effective, with a response rate of 90% and a mean response duration of 34 mo[70]. Hence, the NCCN guidelines recommended avapritinib as first-line therapy for PDGFR D842V-mutant GIST. Among exon 12 and 14 mutants, V561D and N659K are the most common mutations, respectively; both types are sensitive to imatinib. Most GISTs with this mutation are of epithelioid morphology and have relatively good prognosis[71].

Wild-type GISTs: KIT/PDGFRA wild-type GISTs account for approximately 10%-15% of all GISTs. The pathogenesis of wild-type GISTs is unknown, but inactivation of neurofibromatosis type 1 (NF1) and SDH and gain-of-function mutations in genes downstream of KIT and PDGFRA (RAS and BRAF) have been suggested as a possible cause. Mutations in this alternate signaling pathway may lead to primary resistance to imatinib. SDH-deficient GISTs have a higher probability of responding to sunitinib and regorafenib[72], and are considered to have a good prognosis. NF1-related GISTs are multiple and most often located in the small intestine. Histologically, they are of the spindle cell type, contain many stained filamentous fibers and S100-positive cells, have few mitotic figures, and have a relatively good prognosis. NF1-related GISTs may result from related syndromes; up to 25% of NF-1 patients may develop GISTs over their lifetime[73].

BRAF mutations, which are mainly found in melanoma, thyroid papillary carcinoma, and colorectal carcinoma, are localized in exon 15, with valine at position 600 replaced by aspartic acid (V600E). V600E BRAF mutations destabilize the inactive conformation of the BRAF kinase; activated BRAF stimulates the activation of the mitogen-activated protein kinase pathway to induce atypical cell proliferation. This mutation accounts for 4%-13% of GISTs, and are found most frequently in the small intestine, followed by the stomach. The prognosis is relatively good, although they are not highly sensitive to imatinib. The growth of tumors with mutations in BRAF is inhibited by the use of BRAF inhibitors such as dabrafenib, which blocks kinase activity. Dabrafenib has also been reported to have a good therapeutic effect in GIST [74]. Conversely, reports suggest that approximately 50% of patients develop resistance to BRAF inhibitors within 6 mo of initiation of treatment with a single agent[75]. The mechanism of resistance to single-agent BRAF inhibitors is believed reactivation of mitogen-activated protein kinase kinase and extracellular signal-regulated kinase through a bypass pathway, that does not involve BRAF[76]. In malignant melanoma, the combination of BRAF and mitogen-activated protein kinase kinase inhibitors is believed to potently inhibit tumor growth and delay the development of resistance; the same therapeutic effect is expected for GISTs with BRAF mutations.

The impact of KIT, PDGFRA, and BRAF mutational status on the natural history of localized GISTs has been reviewed by Rossi *et al*[77]. They found that GIST patients with KIT mutations had a poorer prognosis than those with PDGFRA mutations or with triple negative (KIT, PDGFRA, and BRAF wild-type) tumors. In addition, they classified GISTs into three molecular risk groups using multivariable Cox regression models. Group I, including KIT exon 13, PDGFRA exon 12, and BRAF mutated GISTs, had the best prognosis. Group II, including KIT exon 17, PDGFRA exon 18 D842V, and PDGFRA exon 14 mutants and triple-negative mutation GISTs, had intermediate prognosis. Group III, including KIT exon 9, exon 11, and PDGFRA exon 18 mutations apart from D842V, had the worst prognosis. These results suggest that genetic mutations have prognostic value and that grouping by mutation is useful in determining the indications of adjuvant therapy; it also complements clinicopathological risk stratification. The features of KIT mutation types are shown in Table 4.

Liquid biopsy

To confirm genetic mutations, and especially secondary mutations, it is necessary to collect tumor tissue. However, if the tumor is located deep in the abdominal cavity owing to recurrence after surgery or bone metastasis, obtaining tumor tissue is challenging. To solve this problem, a liquid biopsy method has been developed for detecting mutations in tumor-related genes using tumor-derived DNA (circulating tumor DNA: ctDNA)[78]. There is a risk of complications from tissue biopsy; in addition, even if a biopsy specimen is used, it is difficult to evaluate the fission image and MIB-1 labeling index of the entire tumor, as the tissue of GIST is not necessarily homogeneous. Liquid biopsy for detecting ctDNA is noninvasive and safe and provides a highly sensitive biomarker. Kang *et al*[79] reported a simple method for detecting primary and secondary mutations in ctDNA from liquid biopsy samples obtained from GIST patients. Additionally, they suggested that these gene mutations could serve as predictive markers for drug resistance. By identifying resistance mutations from plasma DNA, it is possible to increase the dose of imatinib or quickly switch to another drug. In order to apply this method clinically in the future, technical aspects such as reliability and detection sensitivity need to be established.

Drugs other than imatinib

In GIST patients who experience disease progression during imatinib administration, develop secondary resistance, or cannot tolerate imatinib administration, sunitinib and

Table 4 Clinical features of various molecular subtypes of gastrointestinal stromal tumors

Gene mutation	Exon	Proportion	Common mutation	Treatment	Characteristics
KIT	11	70%	Del-inc557/558	Sensitive to imatinib, secondary mutation resistant to sunitinib, some effect for regorafenib	High risk of recurrence
			p.W557_K558 del		Adverse prognosis effect in stomach
			SNSs and Dup		Relatively good prognosis
	9	10%	A502-'503 Dup	Need high dose of imatinib, effective for sunitinib	Mainly in small intestinal, worse prognosis
	13	1%	Lys642Glu	Secondary mutation resistant to imatinib	Mainly in small intestinal
	17	1%	Asn822Lys	Secondary mutation resistant to imatinib and sunitinib, but responding to regorafenib	Mainly in small intestinal
	8	0.30%	Del-Asp419	Sensitive to imatinib	Extragastric, metastatic prone nature
PDGFRA	18	5%	Asp842Val (D842V)	Responds to avapritinib, resistance to imatinib	Mainly in gastric and favorable prognosis
	14	1%	Apn659Lys	Sensitive to imatinib	Relatively good prognosis
	12		V561D	Sensitive to imatinib	Relatively good prognosis
Wild-type GIST		10%-15%	SDH-deficient	Not sensitive to imatinib, response to sunitinib, regorafenib	Overall indolent disease
			NF1	Not sensitive to imatinib, response to sunitinib	Mainly in the small intestine and good prognosis
	15	1%	BRAF	Not sensitive to imatinib, response to dabrafenib	Relatively good prognosis
			K-RAS	Not sensitive to imatinib	

PDGFRA: Platelet derived growth factor receptor; SNSs: Single-nucleotide substitutions; Dup: Duplication; SDH: Succinate dehydrogenase; NF1: Neurofibromatosis type 1.

regorafenib are recommended for second and third-line treatment, respectively. Sunitinib is a multi-targeted TKI inhibitor that targets c-kit, PDGFRA, and vascular endothelial growth factor receptor, thereby inhibiting angiogenesis and cell proliferation. The issue of secondary resistance as well as primary mutations should be taken into account when considering second-line treatment. Sunitinib is active in KIT exon 11 mutations but less effective against GISTs having secondary resistance after imatinib; it is more effective in treating GISTs with exon 9 mutations or of the wild-type. However, sunitinib shows high inhibitory activity against mutations in the ATP-binding site (exon 13); however, its activity is reduced by mutations in the activation-loop region (exons 17 and 18).

Regorafenib is also a multikinase inhibitor for vascular endothelial growth factor receptor 1/2, PDGFR, Kit, BRAF, and RAF and includes mediators that act on angiogenesis and the tumor microenvironment to promote tumor growth. Gene mutations have also been reported to impact the therapeutic effect of regorafenib, which in a genetic search of the primary tumor was found to be particularly effective in patients with metastatic GIST with KIT exon 11 mutations or SDH deficiency[80]. In another study, GIST patients with KIT exon 17 mutations, who had been previously treated with TKI, showed particularly good response to treatment and prolonged PFS[81].

Ripretinib has been recently included in the NCCN guidelines as a fourth-line drug for patients with GIST, whose disease has progressed on imatinib, sunitinib, and regorafenib. This drug is a KIT and PDGFRA inhibitor that blocks initiating KIT mutations 13, 14, 17 and 18; they include KIT D816V and PDGFRA D842V and are expected to show considerable therapeutic effect. Recently, a double-blind randomized placebo-controlled study was conducted in GIST patients with progression on at least imatinib, sunitinib, and regorafenib. In this trial, PFS improved significantly in the group administered ripretinib compared with placebo (6.3 *vs* 1.0 mo, HR = 0.15, $P < 0.0001$); the safety was acceptable[82].

Although TKIs are useful drugs for GIST, their expected effect may not be obtained due to the issue of primary and secondary resistance. Research is therefore ongoing to find new drugs. In recent years, immunotherapy for cancer is gaining popularity, and its therapeutic effect has been clinically proven. Immune checkpoint inhibitors, such as programmed death protein 1 and cytotoxic T-lymphocyte-associated antigen 4, block the transmission of inhibitory signals to maintain T-cell activation and restore anti-tumor effects. Basic research suggests that GISTs with the D842V mutation show immune cells with increased cytolytic activity, and more tumor cells express programmed death protein 1 and programmed death ligand 1[83]. In addition, regulatory T cells and CD8+ T-cells are overexpressed, while the proportion of CD4+ T-cells is low. These data imply that immunotherapy is effective for patients with GIST, especially for those with D842V mutant tumors. The results of several ongoing clinical trials, especially those evaluating combination therapy with other immune therapeutic agents and TKIs are awaited.

CONCLUSION

Laparoscopic surgery and LECS have not only made it possible to ensure complete curative resection in GIST but have also made it possible to perform less invasive surgery aimed at functional preservation. There is also a wider range of available surgical techniques, which may be selected depending on the location and growth pattern of the tumor. It is expected that multimodal treatment with TKIs and surgery will be an option for progressive GISTs and the results of several clinical trials are awaited. Treatment based on genetic information has been established; in the future, novel treatment strategies with newly developed TKIs, molecularly targeted drugs, and immunotherapy may therefore play important roles in the treatment of GIST.

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ORIGINAL ARTICLE

Basic Study

Combined antrum and corpus biopsy protocol improves *Helicobacter pylori* culture success

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Author contributions: McNamara D conceived the study; Brennan DE and Smith SM performed experiments, acquired and analysed data; O'Morain C and McNamara D recruited patients and collected samples; Smith SM prepared the manuscript; all authors critically reviewed the manuscript and approved the final version; Smith SM and McNamara D contributed equally.

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) causes chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Eradication rates have fallen, mainly due to antimicrobial resistance. Consensus guidelines recommend that first-line treatment is based on the local prevalence of antimicrobial resistance and that rescue therapies are guided by antimicrobial susceptibility testing (AST). However, *H. pylori* culture is challenging and culture-based AST is not routinely performed in the majority of hospitals. Optimisation of *H. pylori* culture from clinical specimens will enable more widespread AST to determine the most appropriate antimicrobials for *H. pylori* eradication.

AIM

To determine whether dual antrum and corpus biopsy sampling is superior to single antrum biopsy sampling for *H. pylori* culture.

METHODS

The study received ethical approval from the joint research ethics committee of Tallaght University Hospital and St. James's Hospital. Patients referred for upper gastrointestinal endoscopy were invited to participate. Biopsies were collected in tubes containing Dent's transport medium and patient demographics were recorded. Biopsies were used to inoculate Colombia blood agar plates. Plates were incubated under microaerobic conditions and evaluated for the presence of *H. pylori*. Statistical analyses were performed using Graphpad PRISM. Continuous variables were compared using the two-tailed independent *t*-test. Categorical variables were compared using the two-tailed Fisher exact test. In all cases, a *P* value less than 0.05 was considered significant.

RESULTS

In all, samples from 219 *H. pylori*-infected patients were analysed in the study. The

and hepatology

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mean age of recruited patients was 48 ± 14.9 years and 50.7% (n = 111) were male. The most common endoscopic finding was gastritis (58.9%; n = 129). Gastric ulcer was diagnosed in 4.6% (n = 10) of patients, while duodenal ulcer was diagnosed in 2.7% (n = 6). Single antrum biopsies were collected from 73 patients, whereas combined antrum and corpus biopsies were collected from 146 patients. There was no significant difference in age, sex or endoscopic findings between the two groups. *H. pylori* was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy [64.4% (n = 94/146) *vs* 49.3% (36/73); P = 0.04)].

CONCLUSION

Combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

Key Words: *Helicobacter pylori*; Culture; Antimicrobial susceptibility testing; Antimicrobial; Antrum; Corpus

Core Tip: *Helicobacter pylori (H. pylori)* antimicrobial susceptibility testing is critical to accurately detect antimicrobial resistance, thereby influencing appropriate treatment choices, promoting antimicrobial stewardship and increasing *H. pylori* eradication rates. However, *H. pylori* culture represents a challenge and is limited to a small number of specialized centres and reference laboratories. Increasing biopsy sample number has been suggested to improve culture success, but data directly comparing dual biopsy *vs* single biopsy sample collection for *H. pylori* culture are lacking. Here we show that combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) causes one of the most common bacterial infections globally, colonising the stomach of approximately half of the world's population. This bacterium is of interest clinically as the causative agent of chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. *H. pylori* has been designated a class I carcinogen by the World Health Organisation (WHO)[1]. Treatment usually involves stomach acid suppression using a proton pump inhibitor (PPI) together with 2-3 antimicrobials. However, treatment success has been impacted in recent years, largely due to the emergence of antimicrobial-resistant *H. pylori*. Indeed, the WHO has included *H. pylori* on their priority list of antibiotic-resistant microorganisms[2].

Primary resistance rates for clarithromycin, metronidazole and levofloxacin are 15% or higher in nearly all WHO regions[3]. Recent data on *H. pylori* antimicrobial resistance in European countries revealed overall primary resistance rates of 21.4%, 15.8% and 38.9% for clarithromycin, levofloxacin and metronidazole, respectively[4]. As resistance rates vary from region to region[3-5], consensus guidelines[6-11] recommend that first-line treatment for *H. pylori* is based on primary resistance rates in a given population. If the prevalence of primary clarithromycin resistance is unknown, it is recommended to perform clarithromycin antimicrobial susceptibility testing (AST) before using clarithromycin-based first-line triple therapy. *H. pylori* AST is also recommended to guide rescue therapy following 2 treatment failures[8]. Thus, methods to detect antimicrobial resistance are of great importance both for surveying resistance rates in different regions and for personalising *H. pylori* treatment.

Traditionally, *H. pylori* AST has been performed by culturing the bacteria from stomach tissue biopsies taken during endoscopic examination and determining the minimum inhibitory concentration of an antimicrobial agent required to inhibit bacterial growth[12]. But *H. pylori* is a fastidious organism and culture is challenging and time-consuming with reported success rates varying from 55%-93%[13,14]. Culture success is influenced by many factors, including PPI use, tissue sampling site, choice of transport medium and *H. pylori* growth conditions[4,15]. This study aimed to determine whether a dual antrum and corpus biopsy sampling protocol was superior to a single antrum biopsy protocol for the successful culture of *H. pylori*.

MATERIALS AND METHODS

Study design and ethics

The study was carried out at Tallaght University Hospital, Dublin, Ireland, which is affiliated with Trinity College Dublin. The study received ethical approval from the joint research ethics committee of Tallaght University Hospital and St. James's Hospital. Patients referred for upper gastrointestinal endoscopy were invited to participate. Patients were prospectively recruited to determine the culture success rate when combined antrum and corpus biopsies were used. The culture success rate when single antrum biopsies were used was determined retrospectively.

Study population

Inclusion criteria were (1) Ability and willingness to participate in the study and to provide informed consent; and (2) Confirmed *H. pylori* infection as indicated by a positive rapid urease test (TRI-MED Distributors, PTY LTD, Washington, United States) at 30 min and by histology. Exclusion criteria were (1) Age less than 18 years; (2) Pregnancy or lactation; (3) Severe intercurrent illness; (4) Recent antimicrobial use (within 4 wk); and (5) Bleeding problems or use of blood thinning drugs.

Sample collection

At endoscopy, biopsy samples from each patient were placed directly into collection tubes containing Dent's transport medium [brain heart infusion broth containing 2.5% (w/v) yeast extract, 5% sterile horse serum and *H. pylori* Selective Supplement (Oxoid, Basingstoke, United Kingdom)]. When both antrum and corpus biopsies were collected from a patient, the two tissue samples were placed into the same collection tube. Biopsy samples were processed for culture as soon as possible following endoscopy, usually within 6 h. If processing was delayed, samples were refrigerated at 4 °C and used to inoculate plates within 24 h.

H. pylori culture

The tissue samples were inoculated onto Columbia blood agar plates containing 5% laked horse blood (VWR International, Lutterworth, Leicestershire, United Kingdom) and incubated at 37 °C under microaerobic conditions generated using the CampyGen 2.5 L Atmosphere Generation System (Oxoid). When both antrum and corpus biopsies were collected, they were inoculated onto the same plate. Plates were examined for the presence of *H. pylori* for up to 7 d. *H. pylori* was identified by visual inspection of the colonies, a positive urease test and by polymerase chain reaction.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., CA, United States). Continuous variables are presented as arithmetic mean and standard deviation. Continuous variables were compared using the two-tailed independent *t* test. Categorical variables are presented as percentages and their 95% confidence intervals (95%CI). Categorical variables were compared using the two-tailed Fisher exact test. In all cases, a *P* value less than 0.05 was considered significant.

RESULTS

In all, samples from 219 *H. pylori*-infected patients were analysed. The mean age of recruited patients was 48 ± 14.9 years and 50.7% were male (Table 1). The most common endoscopic finding was gastritis (58.9%; *n* = 129). The rates of more serious *H. pylori*-associated diseases, such as gastric ulcer, duodenal ulcer and intestinal

Table 1 Patient demographics

	Total, *n* = 219	Single, *n* = 73	Combined, *n* = 146	*P* value[1]
Mean age (yr)	48 ± 14.9	49 ± 15.9	48 ± 14.5	0.43
Sex				0.32
Male	*n* = 111 (50.7%)	*n* = 41 (56.2%)	*n* = 70 (47.9%)	
Female	*n* = 108 (49.3%)	*n* = 32 (43.8%)	*n* = 76 (52.1%)	
Endoscopy findings				
Normal	18 (8.2%)	5 (6.8%)	13 (8.9%)	0.80
Gastritis	129 (58.9%)	40 (54.8%)	89 (61.0%)	0.57
Gastric ulcer	10 (4.6%)	3 (4.1%)	7 (4.8%)	1.00
Duodenal ulcer	6 (2.7%)	2 (2.7%)	4 (2.7%)	1.00
Intestinal metaplasia	1 (0.5%)	1 (1.4%)	0 (0%)	0.33
Duodenitis	11 (5.0%)	3 (4.1%)	8 (5.5%)	0.76
Oesophagitis	4 (1.8%)	3 (4.1%)	1 (0.7%)	0.12
Barrett's oesophagus	5 (2.3%)	3 (4.1%)	2 (1.4%)	0.34
Hiatus hernia	9 (4.1%)	3 (4.1%)	6 (4.1%)	1.00
Telangiectasia	1 (0.5%)	0 (0%)	1 (0.7%)	1.00
Portal hypertensive gastropathy	1 (0.5%)	1 (1.4%)	0 (0%)	0.33
No data	24 (11.0%)	9 (12.3%)	15 (10.3%)	0.65

[1]Single versus combined.

metaplasia were low in the study cohort at 4.6% (*n* = 10), 2.7% (*n* = 6) and 0.5% (*n* = 1), respectively (Table 1).

Single antrum biopsies were collected from 73 patients, whereas combined antrum and corpus biopsies were collected from 146 patients. There was no significant difference in age, sex or endoscopic findings between the two groups (Table 1). *H. pylori* was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy [64.4% (*n* = 94/146) *vs* 49.3% (36/73); *P* = 0.04) (Table 2)].

DISCUSSION

H. pylori AST is critical to accurately detect antimicrobial resistance, thereby influencing appropriate treatment choices, promoting antimicrobial stewardship and increasing *H. pylori* eradication rates. While molecular AST methods are available, these are primarily limited to the detection of clarithromycin-and levofloxacin-associated DNA mutations. Culture-based AST remains the only method currently available to test all the antimicrobials potentially useful for *H. pylori* treatment[16]. Despite the importance of culture-based AST , *H. pylori* culture is not routinely performed in the majority of hospitals[5-7,11] either to survey resistance rates or to tailor therapies. From a microbiology perspective, *H. pylori* is challenging to culture. In this study, we report an increased culture success rate when a dual antrum and corpus biopsy protocol was used compared to using a single antrum biopsy (64.4% *vs* 49.3%; *P* = 0.04). While a significant improvement in culture success was observed, a rate of 64.4% is lower than some previous reports. PPI use is known to impact the diagnostic accuracy of *H. pylori* culture[8]. While patients attending for endoscopy at our centre are encouraged to refrain from PPI use 2 wk prior to their scheduled endoscopy, in practice many do not. Nonetheless, the 15% increase in culture success rate reported here provides a strong rationale for a combined biopsy approach.

It is not surprising that the more biopsy specimens used for culture, the higher the chance of recovering *H. pylori* and this practice has been suggested elsewhere[15,17]. However, recent guidelines on the management of *H. pylori*[6-8,11] do not include

Table 2 Culture success rate of *Helicobacter pylori* using single antrum biopsies versus combined antrum and corpus biopsies

	Culture positivity rate	*P* value
Single biopsy	49.3% (36/73; 95%CI: 38.2-60.5)	0.04[a]
Combined biopsies	64.4% (94/146; 95%CI: 56.3-71.7)	

[a]*P* value < 0.05.

specific recommendations on biopsy sampling protocols for *H. pylori* culture and studies directly evaluating culture success using a single *vs* combined biopsy sampling protocol are lacking. The biopsy sampling location is important for a number of reasons. Firstly, collecting biopsies from both the antrum and the corpus takes into account patchy distribution of *H. pylori* in the stomach, which can occur with PPI use [15,18,19]. Furthermore, intragastric location-specific differences in the evolution of *H. pylori* have been reported across strains within the same individual[20]. In terms of AST, it is important to collect biopsies from both sites, as these differences extend to the antimicrobial susceptibility profiles between strains isolated from the corpus and those from the antrum of the same patient[21,22]. Thus, resistance to a given antimicrobial could be missed if biopsy samples from only one location are taken, potentially having a negative impact on treatment outcome.

A limitation of our study is that patients were recruited prospectively to the dual biopsy sampling group, while the single antrum biopsy culture success rate was analysed retrospectively. However, it should be noted that for the entire duration of the patient recruitment and sample collection phases of the study, we followed the standardized protocols of the European *H. pylori* Antimicrobial Susceptibility Testing Working Group[4]. Therefore, the sample transport protocols, microbiological media and culture conditions and methods were consistent throughout the entirety of the study, thereby limiting heterogeneity in this regard.

CONCLUSION

In conclusion, combined corpus and antrum biopsy sampling improves *H. pylori* culture success compared to single antrum biopsy sampling.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (H. pylori) represents a public health issue as the causative agent of chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. Success rates for current therapies have fallen over the years, mainly due to antimicrobial resistance. International guidelines recommend that treatment choices are based on local antimicrobial resistance rates. However, *H. pylori* culture is challenging and culture-based antimicrobial susceptibility testing (AST) is not routinely performed in most healthcare facilities.

Research motivation

Optimisation of *H. pylori* culture from clinical specimens will enable more widespread AST for *H. pylori*.

Research objectives

This research aimed to evaluate biopsy sampling protocols to enhance *H. pylori* culture success, specifically to determine whether dual antrum and corpus biopsy sampling was superior to a single antrum biopsy sampling protocol.

Research methods

Stomach tissue biopsies from rapid-urease test positive patients were collected in tubes containing Dent's transport medium. Biopsies were used to inoculate Colombia blood agar plates. Plates were incubated under microaerobic conditions and evaluated for the presence of *H. pylori*. Culture success rates when a single antrum biopsy was used

were compared to those when dual antrum and corpus biopsies were used.

Research results

H. pylori was successfully cultured in a significantly higher number of cases when combined antrum and corpus biopsies were used compared to a single antrum biopsy sample.

Research conclusions

A combined corpus and antrum biopsy sampling approach improves *H. pylori* culture success compared to a single antrum biopsy sampling protocol.

Research perspectives

Optimisation of *H. pylori* culture methods will encourage more widespread AST. Antimicrobial resistance surveillance is the key to determining the most appropriate antimicrobials for *H. pylori* eradication.

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