ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2019 June 21; 25(23): 2833-2972

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 25 Number 23 June 21, 2019

SYSTEMATIC REVIEWS

META-ANALYSIS

Contents

World Journal of Gastroenterology

Volume 25 Number 23 June 21, 2019

ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Nobuhiro Ohkohchi, MD, PhD, Professor, Department of Surgery, Division of Gastroenterology and Hepatobiliary Surgery and Organ Transplantation, University of Tsukuba, Tsukuba 305-8575, Japan

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, *etc*. The *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

The *WJG* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus and Directory of Open Access Journals. The 2018 edition of Journal Citation Report® cites the 2017 impact factor for *WJG* as 3.300 (5-year impact factor: 3.387), ranking *WJG* as 35th among 80 journals in gastroenterology and hepatology (quartile in category Q2).

RESPONSIBLE EDITORS FOR THIS ISSUE

Responsible Electronic Editor: *Yu-Jie Ma*

Proofing Production Department Director: *Yun-Xiaojian Wu*

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Subrata Ghosh, Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

EDITORIAL OFFICE
Ze-Mao Gong, Director

PUBLICATION DATE
June 21, 2019

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2833-2838

DOI: 10.3748/wjg.v25.i23.2833

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Postoperative complications in gastrointestinal surgery: A "hidden" basic quality indicator

Roberto De la Plaza Llamas, José M Ramia

ORCID number: Roberto de la Plaza Llamas (0000-0001-9501-5480); José M Ramia (0000-0003-1186-953X).

Author contributions: De la Plaza Llamas R and Ramia JM conceived the study; De la Plaza Llamas R design the study; De la Plaza LLamas R performed the reseach; De la Plaza Llamas R and Ramia JM drafted the manuscript; both authors approved the final version of the article.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

Manuscript source: Invited manuscript

Received: March 22, 2019
Peer-review started: March 22, 2019
First decision: April 5, 2019
Revised: April 11, 2019
Accepted: April 19, 2019
Article in press: April 21, 2019
Published online: June 21, 2019

Roberto De la Plaza Llamas, José M Ramia, Department of General and Digestive Surgery, Hospital Universitario de Guadalajara, Guadalajara 19002, Spain

Corresponding author: Roberto De la Plaza Llamas, FACS, MD, MSc, PhD, Associate Professor, Doctor, Medical Assistant, Surgeon, Surgical Oncologist, Department of General and Digestive Surgery, Hospital Universitario de Guadalajara, Calle Donante de sangre s/n, Guadalajara 19002, Spain. rdplazal@sescam.jccm.es
Telephone: +34-94-9209241
Fax: +34-94-9209218

Abstract

Postoperative complications represent a basic quality indicator for measuring outcomes at surgical units. At present, however, they are not systematically measured in all surgical procedures. A more accurate assessment of their impact could help to evaluate the real morbidity associated with different surgical interventions, establish measures for improvement, increase efficiency and identify benchmarking services. The Clavien-Dindo Classification is the most widely used system worldwide for assessing postoperative complications. However, the postoperative period is summarized by the most serious complication without taking into account others of lesser magnitude. Recently, two new scoring systems have emerged, the Comprehensive Complication Index and the Complication Severity Score, which include all postoperative complications and quantify them from 0 (no complications) to 100 (patient's death), These allow the comparison of results. It is important to train surgical staff to report and classify complications and to record 90-d morbidity rates in all patients. Comparisons with other services must take into account patient comorbidities and the complexity of the particular surgical procedure. To avoid subjectivity and bias, external audits are necessary. In addition, ensuring transparency in the reporting of the results is an urgent obligation.

Key words: Morbidity; Postoperative complications; Health policy; Comprehensive Complication Index; Clavien-Dindo Classification; Complication Severity Score

Core tip: Postoperative complications represent a basic quality indicator for measuring surgical outcomes, but at present they are not systematically recorded. A more thorough assessment of their impact could help to determine the real morbidity, establish measures for improvement, increase efficiency and identify benchmarking services. The use of the

P-Reviewer: El-Shabrawi MHF, Kai K, Mann O, Sperti C, Teramoto-Matsubara OT
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

Clavien Dindo Classification of Complications and the Comprehensive Complication Index would allow us to compare them. Surgical staff must be encouraged to report and classify complications and to record 90-d morbidity rates in all patients. External audits are necessary, and ensuring transparency in the reporting of the results is an urgent obligation.

Citation: De la Plaza Llamas R, Ramia JM. Postoperative complications in gastrointestinal surgery: A "hidden" basic quality indicator. *World J Gastroenterol* 2019; 25(23): 2833-2838
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2833.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2833

INTRODUCTION

Postoperative complications represent a basic quality parameter for measuring the results of surgical procedures. Unfortunately, morbidity is not systematically recorded at surgical services. At most, certain services evaluate specific surgeries but for a limited period of time, and usually only the most serious complications are considered. As a consequence, no reliable local or international registries of morbidity are available: The only means available to the scientific community and society at large for assessing the postoperative morbidity associated with particular surgical procedures are isolated studies published by specific surgical services, which are not externally audited and often present better than average results.

Because of the lack of information on the complications associated with particular procedures or at particular units, it is impossible to carry out comparisons with other services, introduce measures for improvement, or learn from other services that obtain better results.

We are accustomed to groups of experts proposing morbidity and mortality standards for performing certain complex surgical procedures. But where do their figures come from? Are their statistics credible? Can they be considered accurate and objective when the services do not record their morbidity?

In cases in which morbidity is recorded, the reporting may be subject to a variety of biases that we will discuss in more detail later. In addition, the reports lack objective certification by an external and fundamentally impartial audit. At most surgical services, these morbidity and mortality standards are considered unattainable. Why is this so? And how can we talk about benchmarking surgical services if we do not know the morbidity associated with each surgical technique at each service, or the real situation in many of the services certified as excellent? Which services are the best, and on what criteria are these qualifications based?

At a time when society demands global transparency, it is hard to explain why this quantification is not mandatory, especially since its consequences have such an important bearing on quality of life, oncological prognosis, and healthcare costs[1,2]. Here, we describe some minimum guidelines designed to allow a process of recording, communication and comparison of postoperative morbidity.

GUIDELINES FOR THE RECORDING, CLASSIFICATION AND COMPARISON OF POSTOPERATIVE COMPLICATIONS

Until recently, it was difficult to quantify postoperative complications because of the lack of any standardized classifications that allowed their systematic recording and comparison.

Martin *et al*[3] conducted a study designed to critically evaluate the quality of surgical literature from 1975 to 2001 in the reporting of complications. They included 119 reports recording outcomes in 22530 patients[3]. Among other things, the authors observed that only 34% of the studies defined the term complication, and that the definitions varied widely (in the case of pancreatic fistula, for instance, they noted up to 12 definitions); only 20% used the degree of severity, and only 67% of the studies indicated the duration of the follow-up. Therefore, the evolution of the methodology for evaluating postoperative morbidity has been heterogeneous, and inconsistent reporting of complications has been a common feature in the surgical literature.

Despite the presence of the tools that we will outline below, in general the des-

criptions of the methodology used in the diagnosis, recording, and monitoring of complications are unsatisfactory: There is a systematic absence of an external and impartial audit, and so the results lack reliability.

In 2004, Dindo *et al*[4] published the classification of complications definitely known as the Classification of Clavien Dindo (CDC)[5], which reached a wide audience. Currently, the article has 10635 citations[6].

The CDC classifies complications in a very intuitive way and was very well received by surgeons, Nonetheless, in complex scenarios their grading may be controversial. For this reason, Clavien's group and others have provided several sets of guidelines[4,5,7-9]. The CDC considers any negative event occurring during hospitalization as a complication[4,5,10,11]. The main problem with this system is that the entire postoperative course is defined according to the most serious complication, and other minor complications are not considered - even though it has been shown that between 44% and 51.5% of patients who present morbidity at surgical services have two complications or more[9,11].

To overcome this problem, in 2013 Slankamenac *et al*[12] developed a new global scoring system for postoperative complications based on the CDC, called the Comprehensive Complication Index (CCI). The CCI evaluates all complications separately according to the CDC; then, after entering them in the online calculator, it rates the morbidity on a scale from 0 to 100, with a score of 0 reflecting the absence of complications and a score of 100 indicating death[12]. The CCI currently has 224 citations[13]; it has been used in 104 published studies and has been discussed in two letters to the editor, two editorials and two comments in PubMed (search updated on March 18, 2019 with the strategy "Comprehensive Complication Index").

In 2015, the Complication Severity Score (CSS) became accessible online. Like the CCI, this system is also based on the CDC and has an overall score of 0 to 100[14]. However, the initial publication describing the scale was rejected[14] and was only finally published in December 2018[15]. The authors claim that it improves on the CCI because the CCI assigns an inappropriately high score to a combination of complications: "*…a patient who develops two Clavien-Dindo grade II complications gets a higher CCI score than a patient who develops a single Clavien-Dindo grade IIIa com-plication…*"[15] . The CSS is similar to the CCI in terms of its elaboration and uses a similar formula, but it assigns less weight to each grade of the CDC.

The CCI and CSS have two obvious advantages over the CDC: They take into account all the complications and produce a composite score, thus allowing the comparison of results.

In a study of all the patients operated upon at a general and digestive surgery service over a one-year period, the CDC and CCI were validated in the four groups of surgical complexity defined by the Operative Severity Score[16], in terms of the following clinical data: Hospital stay, prolongation of hospital stay, readmission and disability[11,17]. The CSS obtained similar results in this series, although the results have not yet been published[17]. The CDC showed slightly lower clinical validation values than the CCI and the CSS[17]. The CCI was the index that was least influenced by confounding factors but in one patient the score exceeded 100, while the CSS did not reach 100 in any case[17], because its numerical values are lower than those of the CCI. Thus, the use of CSS would theoretically have an advantage in highly complex surgeries with a multitude of complications which, in exceptional cases, might produce a higher CCI.

Several studies have shown the relationship between increased costs and higher CDC scores[2,18-21]. Only two teams have studied the relationship of the CCI with postoperative costs. Staiger *et al*[22] reported (among other findings) a strong correlation between the CCI and costs three months post-surgery, and higher correlations for more complex procedures. They also developed a cost prediction tool. De la Plaza *et al*[2] studied the postoperative costs in patients operated over a one-year period at a general surgery and digestive service, finding a moderate to strong correlation of the CCI with overall postoperative costs, which increased with the surgical complexity according to the Operative Severity Score. In all the groups, this correlation was higher in emergency surgery. In addition, the CCI was correlated with postoperative costs in patients with prolonged postoperative stay and in those without, and also with the initial operating room costs[2]. This relationship between postoperative costs and the CCI provides further support for the score's clinical validity.

Comparing the two new systems, we believe that the CCI should be preferred to the CSS for the following reasons: (1) The preparation of the CCI involved 227 patients and 245 physicians (surgeons, anesthetists and intensivists)[12]. In the preparation of the CSS, only 49 senior gastrointestinal and hepato-pancreatico-biliary surgeons in India were included ("senior" being defined as having at least 5 years of experience after graduating), and no patients[15,23]; and (2) The CCI has a greater

diffusion worldwide[13].

A summary of these three tools (CDC, CCI and CSS) is shown in Table 1.

Therefore, the CDC is the most suitable classification for each individual postoperative complication, while the CCI is able to numerically quantify the postoperative complications in a particular patient or group of patients undergoing a surgical procedure. In addition, the percentage of certain important complications is also specified for each surgical procedure: For example, in esophagectomies, gastrectomies, pancreatectomies, colectomies, and so on, the presence of specific and important complications such as the presence of anastomosis and pancreatic fistulas is recorded. In any case, the mere fact of initially classifying each complication according to the CDC would alert surgical departments to less important complications that are relatively easy to improve, such as infection of the surgical wound, urinary tract infection, central venous catheter, or pulmonary complications.

Complications should be recorded for at least 90 d post-surgery, as should readmissions in that period. Between 30 and 90 d postoperatively the number of complications rises by 11.6%[17]. The recording of complications that occur outside the hospital environment is a more difficult issue. These complications are less serious, but it is important to evaluate them (for example, complications after less complex procedures such as cholecystectomy). This problem could be minimized by the use of an electronic medical recording system that incorporates the care carried out outside the hospital.

It is essential to report complications as they occur, or at least when evidence of the event becomes available. The event should be recorded in the patient progress notes or in specially designed forms in which the complication and the treatment are reported in writing and the CDC. Consultation of nurses' notes is also fundamental.

Within 90 d of the procedure, a summary of the morbidity in each patient should be made by the physicians at the service based on the clinical history, and should be stored in (for example) an Excel table recording each complication, the CDC, and the CCI[11]. In our experience, the average time taken to evaluate complications at 90 d post-surgery and to record them in the spreadsheet ranges between 5 and 10 min per patient.

To compare the results at different services, one must take into account the complexity of the patient's condition, not just on the basis of the ASA but by making a risk adjustment with complexity or severity scores such as the Charlson Comorbidity scale[24]. It is also important to compare surgical procedures of similar complexity and technical difficulty[2]. An impartial external audit is essential. When used by physicians at our service to record morbidity and applying the methodology described above, the CDC and CCI presented accuracy rates of 88% and 81%; however, when only patients with complications were included, the rates fell respectively to 69% and 49%[17,25].

There are several explanations for the fact that postoperative complications are only rarely quantified. The most important, in our view, is the fact that the better the recording system, the worse the results. Surgeons may regard complications as an indication of personal failure, and fear comparison with other services because the results may reflect badly on their work. Furthermore, at a time when centres of reference are being established for complex surgical procedures such as esophagectomy, gastrectomy, pancreatectomy or hepatectomy, high morbidity rates at particular services might disqualify them from operating on these patients.

It is hard to understand why public authorities choose these services of reference only on the basis of volume (or sometimes for political reasons) and fail to take actual audited results into account, such as morbidity and disease-free survival in cancer patients. Besides questions of cost-effectiveness, ensuring the optimal use of the means available is an obligation in a system with limited resources.

Another common bias is the failure to record certain minor complications. For example, the CDC includes nausea, poorly controlled pain and atelectasis as complications, which in practice may go unreported. The inclusion of minor complications may magnify the actual morbidity, but it eliminates subjective interpretations and makes them the same for all auditors. The presence of errors in the classification of complications according to the CDC, particularly in complex scenarios, should also be borne in mind. However, many useful clarifications have already been made in this regard[4,5,7-9].

CONCLUSION

Little is known at present about real postoperative morbidity.

In the recording of postoperative complications, the following points must be taken

Table 1 Comparison of the characteristics of the clavien dindo classification, the comprehensive complication index and the complication severity score

	CDC	CCI	CSS
Year of publication	2004	2013	2018
Criteria used	Opinions of 144 surgeons	Opinions of 227 patients and 245 physicians (surgeons, anesthetists and intensivists)	Opinions of 49 gastrointestinal and hepato-pancreatico-biliary surgeons in India
Scale and calculation	Classifies the complications in 5 grades. The therapy used to correct a specific complication remains the cornerstone to rank a complication.	All the complications must be classified according to the CDC, and the score is then calculated with the formula or on-line.	All the complications must be classified according to the CDC, and the score is then calculated with the formula.
Value	Considers only the most severe complication: 0-V	Considers all the complications: 0-100. Higher numerical value than the CSS.	Considers all the complications: 0-100. Lower numerical value than the CCI
Validation with clinical results	Yes	Yes	Yes (PhD thesis)[17]
Bibliographical citings	10635	224	2
Management	More straightforward	More complex in patients with multiple complications	More complex in patients with multiple complications
Does it adequately represent the postoperative course of patients with ≥ 2 complications?	No	Yes	Yes
Does it all comparison of the results?	No, if there are ≥ 2 complications	Yes	Yes

CDC: Clavien Dindo Classification; CSS: Complication Severity Score; CCI: Comprehensive Complication Index.

into account: (1) A complication should be considered as any negative event occurring in a patient during hospitalization[4,5,10,11]; (2) Physicians and nurses must be made aware of the need to record complications; (3) Training with the CDC must be provided, especially in order to deal with complex scenarios; (4) Exhaustive, external impartial recording of all complications must be performed. However, biases cannot be totally avoided since the registry is performed by doctors and nurses at the service under evaluation; (5) Complications should be recorded on a form specially created for the purpose; (6) Physicians' and nurses' notes should be consulted; and (7) An external audit must be carried out by experts without any conflict of interest with regard to the surgery service or its members so as to avoid deficiencies in the recording and classification and other biases.

The recording of the complications deriving from all surgical procedures is an urgent scientific and social obligation. Transparency in the reporting is also mandatory. There are sufficient means available now to record complications accurately and efficiently, with only minimal investment and the results are available in the short-medium term. Policy-makers in the field of health administration should not let this opportunity pass.

REFERENCES

1 **Slankamenac K**, Slankamenac M, Schlegel A, Nocito A, Rickenbacher A, Clavien PA, Turina M. Impact of postoperative complications on readmission and long-term survival in patients following surgery for colorectal cancer. *Int J Colorectal Dis* 2017; **32**: 805-811 [PMID: 28411352 DOI: 10.1007/s00384-017-2811-y]

2 **de la Plaza Llamas R**, Hidalgo Vega Á, Latorre Fragua RA, López Marcano AJ, Medina Velasco AA, Díaz Gil JM, Ramia Ángel JM. The Cost of Postoperative Complications and Economic Validation of the Comprehensive Complication Index. *Ann Surg* 2019; 1 [PMID: 30985367 DOI: 10.1097/SLA.0000000000003308]

3 **Martin RC 2nd**, Brennan MF, Jaques DP. Quality of complication reporting in the surgical literature. *Ann Surg* 2002; **235**: 803-813 [PMID: 12035036 DOI: 10.1097/00000658-200206000-00007]

4 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542 DOI: 10.1097/01.sla.0000133083.54934.ae]

5 **Clavien PA**, Barkun J, de Oliveira ML, Vauthey JN, Dindo D, Schulick RD, de Santibañes E, Pekolj J, Slankamenac K, Bassi C, Graf R, Vonlanthen R, Padbury R, Cameron JL, Makuuchi M. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; **250**: 187-196 [PMID: 19638912 DOI: 10.1097/SLA.0b013e3181b13ca2]

6 Classification of Surgical Complications Overview of attention for article published in Annals of Surgery, August 2004 [Internet]. Wolters Kluwer, Artic. Metrics [cited 2019 Mar 18]. Available from: https://wolterskluwer.altmetric.com/details/4800046/citations

7 **Mentula PJ**, Leppäniemi AK. Applicability of the Clavien-Dindo classification to emergency surgical procedures: a retrospective cohort study on 444 consecutive patients. *Patient Saf Surg* 2014; **8**: 31 [PMID: 25075222 DOI: 10.1186/1754-9493-8-31]

8 **Katayama H**, Kurokawa Y, Nakamura K, Ito H, Kanemitsu Y, Masuda N, Tsubosa Y, Satoh T, Yokomizo A, Fukuda H, Sasako M. Extended Clavien-Dindo classification of surgical complications: Japan Clinical Oncology Group postoperative complications criteria. *Surg Today* 2016; **46**: 668-685 [PMID: 26289837 DOI: 10.1007/s00595-015-1236-x]

9 **Clavien PA**, Vetter D, Staiger RD, Slankamenac K, Mehra T, Graf R, Puhan MA. The Comprehensive Complication Index (CCI®): Added Value and Clinical Perspectives 3 Years "Down the Line". *Ann Surg* 2017; **265**: 1045-1050 [PMID: 28486288 DOI: 10.1097/SLA.0000000000002132]

10 **Clavien PA**, Sanabria JR, Strasberg SM. Proposed classification of complications of surgery with examples of utility in cholecystectomy. *Surgery* 1992; **111**: 518-526 [PMID: 1598671]

11 **de la Plaza Llamas R**, Ramia Ángel JM, Bellón JM, Arteaga Peralta V, García Amador C, López Marcano AJ, Medina Velasco AA, González Sierra B, Manuel Vázquez A. Clinical Validation of the Comprehensive Complication Index as a Measure of Postoperative Morbidity at a Surgical Department: A Prospective Study. *Ann Surg* 2018; **268**: 838-844 [PMID: 30303875 DOI: 10.1097/SLA.0000000000002839]

12 **Slankamenac K**, Graf R, Barkun J, Puhan MA, Clavien PA. The comprehensive complication index: a novel continuous scale to measure surgical morbidity. *Ann Surg* 2013; **258**: 1-7 [PMID: 23728278 DOI: 10.1097/SLA.0b013e318296c732]

13 The Comprehensive Complication Index Overview of attention for article published in Annals of Surgery, July 2013 [Internet]. Wolters Kluwer, Artic. Metrics [cited 2019 Mar 18]. Available from: https://wolterskluwer.altmetric.com/details/1525609/citations

14 **Panwar R**, Sahni P. Retracted: Measuring Postoperative Complications: On the Right Path but Far Away From the Destination. *Ann Surg* 2017 [PMID: 27140508 DOI: 10.1097/SLA.0000000000001616]

15 **Panwar R**, Mohapatra V, Raichurkar K, Sahni P. Development and validation of a new score for measuring post-operative complications. *Langenbecks Arch Surg* 2018; **403**: 1021-1027 [PMID: 30094627 DOI: 10.1007/s00423-018-1701-2]

16 **Copeland GP**, Jones D, Walters M. POSSUM: a scoring system for surgical audit. *Br J Surg* 1991; **78**: 355-360 [PMID: 2021856 DOI: 10.1002/bjs.1800780327]

17 **de la Plaza Llamas R**. Validación y aplicabilidad clínica del Comprehensive Complication Index en una población de pacientes intervenidos en un servicio de cirugía general y del aparato digestivo. Estudio prospectivo. Thesis, University of Alcalá. 2018; Available from: https://www.educacion.gob.es/teseo/mostrarRef.do?ref=444441

18 **Vonlanthen R**, Slankamenac K, Breitenstein S, Puhan MA, Muller MK, Hahnloser D, Hauri D, Graf R, Clavien PA. The impact of complications on costs of major surgical procedures: a cost analysis of 1200 patients. *Ann Surg* 2011; **254**: 907-913 [PMID: 21562405 DOI: 10.1097/SLA.0b013e31821d4a43]

19 **Straatman J**, Cuesta MA, de Lange-de Klerk ES, van der Peet DL. Hospital cost-analysis of complications after major abdominal surgery. *Dig Surg* 2015; **32**: 150-156 [PMID: 25791798 DOI: 10.1159/000371861]

20 **Goense L**, van Dijk WA, Govaert JA, van Rossum PS, Ruurda JP, van Hillegersberg R. Hospital costs of complications after esophagectomy for cancer. *Eur J Surg Oncol* 2017; **43**: 696-702 [PMID: 28012715 DOI: 10.1016/j.ejso.2016.11.013]

21 **Wang J**, Ma R, Eleftheriou P, Churilov L, Debono D, Robbins R, Nikfarjam M, Christophi C, Weinberg L. Health economic implications of complications associated with pancreaticoduodenectomy at a University Hospital: a retrospective cohort cost study. *HPB (Oxford)* 2018; **20**: 423-431 [PMID: 29248401 DOI: 10.1016/j.hpb.2017.11.001]

22 **Staiger RD**, Cimino M, Javed A, Biondo S, Fondevila C, Périnel J, Aragão AC, Torzilli G, Wolfgang C, Adham M, Pinto-Marques H, Dutkowski P, Puhan MA, Clavien PA. The Comprehensive Complication Index (CCI®) is a Novel Cost Assessment Tool for Surgical Procedures. *Ann Surg* 2018; **268**: 784-791 [PMID: 30272585 DOI: 10.1097/SLA.0000000000002902]

23 **Staiger RD**, Puhan MA, Clavien PA. Editorial comment: different perspectives on severity of postoperative morbidity. *Langenbecks Arch Surg* 2018; **403**: 1029-1030 [PMID: 30607531 DOI: 10.1007/s00423-018-1738-2]

24 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383 [PMID: 3558716]

25 **de la Plaza Llamas R**, Ramia Ángel J, García Gil J, Arteaga Peralta V, García Amador C, López Marcano A, Medina Velasco A, Díaz Candelas D. Registro prospectivo de todas las complicaciones postoperatorias en un servicio de cirugía general. ¿Como hacerlo? *Cir Esp* 2018; **96** Suppl: S7 Available from: http://www.elsevier.es/es-revista-cirugia-espanola-36-congresos-xxxii-congreso-nacional-cirugia-77-sesion-gestion-de-calidad-4863-comunicacion-registro-prospectivo-de-todas-las-55950-pdf

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2839-2845

DOI: 10.3748/wjg.v25.i23.2839

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FIELD OF VISION

Modified FOLFIRINOX for resected pancreatic cancer: Opportunities and challenges

Feng Yang, Chen Jin, De-Liang Fu, Andrew L Warshaw

ORCID number: Feng Yang (0000-0001-8790-6072); Chen Jin (0000-0002-4330-9716); De-Liang Fu (0000-0003-4060-8101); Andrew L Warshaw (0000-0001-7788-3110).

Author contributions: Yang F collected the materials, discussed the topic, wrote the manuscript, and supervised the publication of this commentary; Jin C and Fu DL discussed the topic; and Warshaw AL discussed the topic and revised the manuscript.

Supported by the National Key R&D Program of China, No. 2017YFC1308604.

Conflict-of-interest statement: We declare no conflicts of interest.

Manuscript source: Invited manuscript

Received: March 7, 2019
Peer-review started: March 7, 2019
First decision: April 5, 2019
Revised: April 17, 2019
Accepted: April 29, 2019

Feng Yang, Chen Jin, De-Liang Fu, Department of Pancreatic Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China

Andrew L Warshaw, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, United States

Corresponding author: Feng Yang, MD, PhD, Associate Professor, Surgeon, Surgical Oncologist, Department of Pancreatic Surgery, Pancreatic Disease Institute, Huashan Hospital, Shanghai Medical College, Fudan University, 12 Central Urumqi Road, Shanghai 200040, China. yffudan98@126.com
Telephone: +86-21-52887164
Fax: +86-21-52888277

Abstract

Pancreatic cancer is one of the leading causes of cancer death worldwide. Adjuvant chemotherapy has been developed based on the experiences made with palliative chemotherapy, and advocated to improve long-term survival of patients with this disease. However, the optimal chemotherapeutic regimen remains controversial. Recently, Conroy *et al* demonstrated the impressive benefits of modified FOLFIRINOX over gemcitabine alone in the multicenter Partenariat de Recherche en Oncologie Digestive 24 (PRODIGE-24) trial. The remarkable results mark a new milestone in treating resectable pancreatic cancer and have now changed the standard of care for this patient population. In this commentary, we discuss an issue of difference of tumor grade between the PRODIGE-24 trial and previous phase III trials. We also discuss potential biomarkers predicting therapeutic response to modified FOLFIRINOX. Finally, we summarize several ongoing clinical trials of replacing part of the FOLFIRINOX regimen with Xeloda/S-1/nanoliposomal irinotecan for pancreatic cancer.

Key words: Pancreatic cancer; Adjuvant therapy; FOLFIRINOX; Neutrophil-to-lymphocyte; Nanoliposomal irinotecan

Core tip: Adjuvant chemotherapy for pancreatic cancer has been developed based on the experiences made with palliative chemotherapy. Nevertheless, the optimal regimen remains controversial. The Partenariat de Recherche en Oncologie Digestive 24 trial showed an impressive benefit of modified FOLFIRINOX over gemcitabine alone. The

Article in press: April 29, 2019
Published online: June 21, 2019

P-Reviewer: Kleeff J, Kopljar M, Neri V
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

remarkable results mark a new milestone in treating resectable pancreatic cancer and have now changed the standard of care. Here, we discuss an issue of difference between this trial and previous phase III trials, as well as potential biomarkers predicting therapeutic response. Several ongoing trials of replacing part of the FOLFIRINOX regimen with other drugs for pancreatic cancer are also summarized.

Citation: Yang F, Jin C, Fu DL, Warshaw AL. Modified FOLFIRINOX for resected pancreatic cancer: Opportunities and challenges. *World J Gastroenterol* 2019; 25(23): 2839-2845
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2839.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2839

COMMENTARY ON HOT TOPICS

We have read with great interest the recent article by Conroy *et al*[1] comparing the efficacy and safety of a modified FOLFIRINOX (oxaliplatin 85 mg/m^2, leucovorin 400 mg/m^2, irinotecan 150 mg/m^2, and continuous fluorouracil 2400 mg/m^2; without bolus fluorouracil; every 2 wk for 12 cycles) regimen with gemcitabine as adjuvant therapy for resected pancreatic cancer, and would strongly recommend it to the readers.

Pancreatic cancer is one of the leading causes of cancer death in both the United States and China[2,3]. The improvement of long-term prognosis of this disease is attributed to the decrease of perioperative mortality[4,5] and the increase of adjuvant treatment rate[6]. Adjuvant chemotherapy has been developed based on the experiences made with palliative chemotherapy, and advocated to reduce recurrence and improve survival after surgery[7]. The European Study Group for Pancreatic Cancer 1 (ESPAC-1) trial revealed a superior median survival with chemotherapy (bolus fluorouracil 425 mg/m^2 plus leucovorin 20 mg/m^2 days 1-5, every 4 wk for six cycles) as compared with no chemotherapy [20.1 mo *vs* 15.5 mo; hazard ratio (HR) for death = 0.71; 95% confidence interval (CI): 0.55-0.92; P = 0.009][8]. The Charité Onkologi 001 (CONKO-001) trial showed a significantly prolonged disease-free survival in the gemcitabine group (1000 mg/m^2 days 1, 8, and 15, every 4 wk for six cycles) compared to the observation group (13.4 mo *vs* 6.9 mo, P < 0.001), but a modest overall survival benefit (22.1 mo *vs* 20.2 mo, P = 0.06)[9]. The ESPAC-3 trial subsequently showed an absence of overall survival difference between adjuvant gemcitabine and fluorouracil plus folinic acid (23.6 mo *vs* 23 mo, P = 0.39) in patients with resected pancreatic cancer[10]. With the goal to further prolong postoperative survival, gemcitabine-based combination chemotherapies were evaluated in subsequent trials, such as the ESPAC-4[11] and CONKO-005 trials[12]. However, the optimal chemotherapeutic regimen remains controversial.

In the Partenariat de Recherche en Oncologie Digestive 24 (PRODIGE-24) trial[1], 493 patients from 77 centers in France and Canada were randomly assigned to receive a modified FOLFIRINOX regimen or gemcitabine for 24 wk. Patients 18 to 79 years of age who had undergone R0 (absence of tumor cells within 1 mm of all resection margins) or R1 (presence of tumor cells within 1 mm of one or more resection margins) resection within 3 to 12 wk before randomization, and who had a serum CA 19-9 level ≤ 180 U/mL were eligible for inclusion. At a median follow-up of 33.6 mo, there was an impressive difference of 18.3% in the disease-free 3-year survival rate (39.7% in the modified FOLFIRINOX group *vs* 21.4% in the gemcitabine group). The median disease-free survival with the modified FOLFIRINOX regimen was 21.6 mo and with gemcitabine 12.8 mo (stratified HR for cancer-related event, second cancer, or death = 0.58; 95%CI: 0.46-0.73; P < 0.001). The median overall survival was 54.4 mo in the modified FOLFIRINOX group *vs* 35.0 mo in the gemcitabine group (stratified HR for death = 0.64; 95%CI: 0.48-0.86; P = 0.003). Compared with previous phase III trials[9-13], the PRODIGE-24 trial showed a much longer median overall survival in the gemcitabine group (Figure 1), which the authors attributed to the high use of FOLFIRINOX and other active regimens after tumor relapse. Kindler[14] considered that the similarity of the disease-free survival in the gemcitabine group throughout these trials argued against selection bias. However, we propose that the difference of tumor grade among these trials should not be neglected (Table 1). Tumor grade has been revealed as an independent prognostic factor for overall survival[10,15]. The higher proportion of well-differentiated tumors might account for the improved survival of

the gemcitabine group in the PRODIGE-24 trial.

A multicenter study from Europe revealed that the overall survival rates of different chemotherapeutic regimens in real-life settings were lower than those shown in randomized phase III trials[16]. This can be explained by the fact that randomized clinical trials usually have stringent inclusion criteria. Predictive markers investigating therapeutic response to modified FOLFIRINOX would help us guide treatment strategies and improve prognosis of pancreatic cancer. Meanwhile, given the more reported incidences of adverse events in the modified FOLFIRINOX group, it is urgently needed to identify patients who will most likely benefit from this regimen. Most recent studies failed to establish tolerability of UGT1A1 genotype-guided modified FOLFIRINOX in pancreatic cancer[17,18]. Interestingly, although the rates of neutropenia and severe lymphopenia were similar between the two groups in the PRODIGE-24 trial[1], the significantly lower occurrence of lymphopenia (any grade) in the modified FOLFIRINOX group indicates a difference of post-treatment change in neutrophil-to-lymphocyte ratio (NLR). NLR, a marker of systemic in-flammatory response, has been shown to be a valuable prognostic marker in many malignancies, including pancreatic cancer[19,20]. Baseline and post-treatment NLR may predict therapeutic response to chemotherapy[21-24], including the modified FOLFIRINOX regimen[25], for pancreatic cancer. In their study, Conroy *et al*[1] showed the treatment benefit favoring modified FOLFIRINOX over gemcitabine in all predefined subgroup analyses. Nevertheless, whether the superiority of the modified FOLFIRINOX regimen is based on difference in the baseline and post-chemotherapy NLR remains unclear, which encourages us to investigate how NLR correlates with response to modified FOLFIRINOX.

The remarkable results of the PRODIGE-24 trial mark a new milestone in treating resectable pancreatic cancer and open a world of possible investigations. Can we further improve survivals by using modified FOLFIRINOX as neoadjuvant chemotherapy? What is the effect of this regimen combined with radiotherapy? Additionally, can we improve the safety and/or efficacy by replacing 5-FU and leucovorin in the FOLFIRINOX regimen with capecitabine (Xeloda) or oral S-1? The triple combination chemotherapy of S-1, oxaliplatin, and irinotecan (SOXIRI) appeared to be a promising and well-tolerated regimen in patients with unresectable pancreatic ductal adenocarcinoma[26,27]. Several phase I and II trials of these particular combinations have been conducted across various stages of pancreatic cancer (Table 2). Lastly, given the theoretical benefits of developing nano-formulations of anti-cancer drugs for cancers[28,29], the applicability of novel agents needs to be evaluated in pancreatic cancer. For example, the proven efficacy of nanoliposomal irinotecan (nal-IRI) in metastatic pancreatic cancer[30,31] has triggered enthusiasm in substituting nal-IRI for standard irinotecan as part of the FOLFIRINOX regimen (Table 2). We look forward to the results of this triple-drug combination regimen in patients with pancreatic cancer.

The modified FOLFIRINOX regimen is superior to gemcitabine as adjuvant therapy for resected pancreatic cancer, and should be a new standard of care for this patient population. It is worth noting that both modified FOLFIRINOX and nab-paclitaxel plus gemcitabine have been established as standard first-line treatment for metastatic pancreatic cancer, showing comparable efficacy outcomes[32]. The APACT trial is a phase III, international, multicenter, randomized, open-label, controlled study to compare adjuvant nab-paclitaxel plus gemcitabine *vs* gemcitabine alone in patients with surgically resected pancreatic cancer (ClinicalTrials.gov, NCT01964430). Eight hundred and sixty-six patients have been randomized in a 1:1 ratio to receive six cycles of either nab-paclitaxel 125 mg/m^2 plus gemcitabine 1000 mg/m^2 or gemcitabine alone 1000 mg/m^2 on days 1, 8, and 15 of a 4-wk cycle. The preliminary results will be announced in the near future, and provide us further evidence for adjuvant treatment for resected pancreatic cancer.

Neoadjuvant treatment with FOLFIRINOX or gemcitabine plus nab-paclitaxel has become a standard of care for borderline or locally advanced pancreatic cancer[33,34], and is now increasingly considered even for up-front resectable disease[35-38]. Due to its growing preference over either postoperative chemotherapy regimen, a neoadjuvant approach is advocated when there is the option in order to improve the success of complete tumor resection and for potential control of micrometastases. Studies now in progress will be critical not only to assess the long-term outcomes of current neoadjuvant regimens but also to investigate the added efficacy of anti-stromal agents such as losartan, immunotherapy, radiation therapy, and biomarkers reflecting the response to treatment.

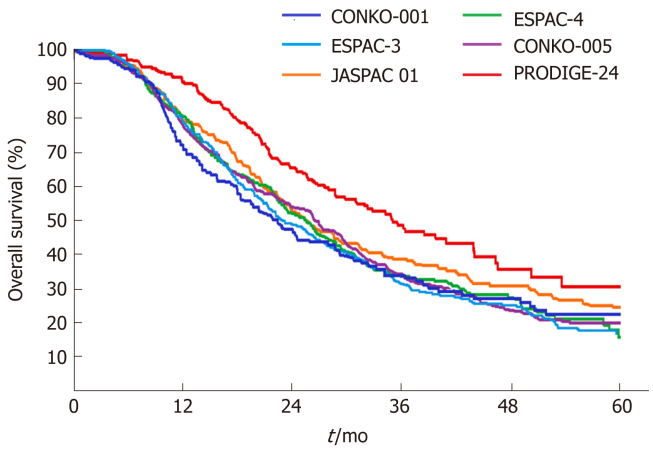
Table 1 Comparison of six phase III clinical trials of gemcitabine alone in patients with resected pancreatic cancer

Trial	PRODIGE-24[1]	CONKO-001[9]	ESPAC-3[10]	JASPAC 01[13]	ESPAC-4[11]	CONKO-005[12]
Variable						
No. of patients	246	179	537	190	366	217
Tumor grade, *n* (%)						
Well differentiated	79 (32.1)	10 (5.6)	66 (12.3)	NA	30 (8.2)	9 (4.1)
Moderately differentiated	125 (50.8)	103 (57.5)	336 (62.6)	NA	192 (52.5)	128 (59)
Poorly differentiated/undifferentiated	29 (11.8)	63 (35.2)	127 (23.6)	NA	142 (38.8)	74 (34.1)
Disease free survival - mo						
Median	12.8	13.4	14.3	11.3	13.1	11.4
95%CI	11.7-15.2	11.4-15.3	13.5-15.6	9.7-13.6	11.6-15.3	9.2-13.6
Overall survival - mo						
Median	35.0	22.1	23.6	25.5	25.5	26.5
95%CI	28.7-43.9	18.4-25.8	21.4-26.4	22.5-29.6	22.7-27.9	22.4-30.6

NA: Not available; CI: Confidence interval.

Table 2 Ongoing clinical trials of replacing part of the FOLFIRINOX regimen with Xeloda/S-1/Nanoliposomal Irinotecan for pancreatic cancer registered in ClinicalTrials.gov

Country	Title	Regimen	Phase	Cancer stage	Estimated enrollment	Study completion date	Source
China	The combination chemotherapy of S-1, irinotecan, and oxaliplatin as first line chemotherapy for pancreatic cancer	S-1, irinotecan, and oxaliplatin	II	Unresectable or metastatic	65	December 2019	NCT03403101
United States	Neoadjuvant capecitabine, oxaliplatin, and irinotecan chemotherapy in the treatment of pancreatic adenocarcinoma	Capecitabine, oxaliplatin, and irinotecan	II	Resectable, borderline and locally advanced	17	December 2022	NCT01760252
Singapore	Oxaliplatin, Xeloda, and irinotecan in pancreatic adenocarcinoma	Oxaliplatin, Xeloda, and irinotecan	I	Advanced and/or metastatic	90	June 2019	NCT02368860
Italy	A study of nanoliposomal irinotecan with 5-fluorouracil, levofolinic acid, and oxaliplatin in patients with resectable pancreatic cancer	Nanoliposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil	II	Resectable	67	January 2020	NCT03528785
United States	Study of nanoliposomal irinotecan-containing regimens in patients with previously untreated, metastatic pancreatic adenocarcinoma	Nanoliposomal irinotecan, oxaliplatin, leucovorin, and 5-fluorouracil	II	Metastatic	56	February 2020	NCT02551991



No. at risk						
CONKO-001	179	128	73	36	23	14
ESPAC-3	537	415	251	103	42	13
JASPAC 01	190	151	100	73	58	45
ESPAC-4	366	302[1]	207[2]	61[3]	27[4]	9
CONKO-005	217	167	113	63	31	19
PRODIGE-24	246	215	120	55	18	4

Figure 1 Overall survival curves for patients receiving gemcitabine alone following pancreatic cancer resection in six phase III randomized clinical trials. [1]Data for the 10th month; [2]Data for the 20th month; [3]Data for the 40th month; [4]Data for the 50th month.

REFERENCES

1 **Conroy T**, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, Ychou M, Volet J, Sauvanet A, Breysacher G, Di Fiore F, Cripps C, Kavan P, Texereau P, Bouhier-Leporrier K, Khemissa-Akouz F, Legoux JL, Juzyna B, Gourgou S, O'Callaghan CJ, Jouffroy-Zeller C, Rat P, Malka D, Castan F, Bachet JB; Canadian Cancer Trials Group and the Unicancer-GI–PRODIGE Group. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2395-2406 [PMID: 30575490 DOI: 10.1056/NEJMoa1809775]

2 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019; **69**: 7-34 [PMID: 30620402 DOI: 10.3322/caac.21551]

3 **Lin QJ**, Yang F, Jin C, Fu DL. Current status and progress of pancreatic cancer in China. *World J Gastroenterol* 2015; **21**: 7988-8003 [PMID: 26185370 DOI: 10.3748/wjg.v21.i26.7988]

4 **Yang F**, Jin C, Li J, Di Y, Zhang J, Fu D. Clinical significance of drain fluid culture after pancreaticoduodenectomy. *J Hepatobiliary Pancreat Sci* 2018; **25**: 508-517 [PMID: 30328297 DOI: 10.1002/jhbp.589]

5 **Yang F**, Jin C, Hao S, Fu D. Drain Contamination after Distal Pancreatectomy: Incidence, Risk Factors, and Association with Postoperative Pancreatic Fistula. *J Gastrointest Surg* 2019 [PMID: 30815778 DOI: 10.1007/s11605-019-04155-7]

6 **Klaiber U**, Leonhardt CS, Strobel O, Tjaden C, Hackert T, Neoptolemos JP. Neoadjuvant and adjuvant chemotherapy in pancreatic cancer. *Langenbecks Arch Surg* 2018; **403**: 917-932 [PMID: 30397779 DOI: 10.1007/s00423-018-1724-8]

7 **Liao WC**, Chien KL, Lin YL, Wu MS, Lin JT, Wang HP, Tu YK. Adjuvant treatments for resected pancreatic adenocarcinoma: a systematic review and network meta-analysis. *Lancet Oncol* 2013; **14**: 1095-1103 [PMID: 24035532 DOI: 10.1016/S1470-2045(13)70388-7]

8 **Neoptolemos JP**, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, Beger H, Fernandez-Cruz L, Dervenis C, Lacaine F, Falconi M, Pederzoli P, Pap A, Spooner D, Kerr DJ, Büchler MW; European Study Group for Pancreatic Cancer. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med* 2004; **350**: 1200-1210 [PMID: 15028824]

9 **Oettle H**, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, Schramm H, Fahlke J, Zuelke C, Burkart C, Gutberlet K, Kettner E, Schmalenberg H, Weigang-Koehler K, Bechstein WO, Niedergethmann M, Schmidt-Wolf I, Roll L, Doerken B, Riess H. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA* 2007; **297**: 267-277 [PMID: 17227978]

10 **Neoptolemos JP**, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, Padbury R, Moore MJ, Gallinger S, Mariette C, Wente MN, Izbicki JR, Friess H, Lerch MM, Dervenis C, Oláh A, Butturini G, Doi R, Lind PA, Smith D, Valle JW, Palmer DH, Buckels JA, Thompson J, McKay CJ, Rawcliffe CL, Büchler MW; European Study Group for Pancreatic Cancer. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *JAMA* 2010; **304**: 1073-1081 [PMID: 20823433 DOI: 10.1001/jama.2010.1275]

11 **Neoptolemos JP**, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, Faluyi O, O'Reilly DA, Cunningham D, Wadsley J, Darby S, Meyer T, Gillmore R, Anthoney A, Lind P, Glimelius B, Falk S, Izbicki JR, Middleton GW, Cummins S, Ross PJ, Wasan H, McDonald A, Crosby T, Ma YT, Patel K, Sherriff D, Soomal R, Borg D, Sothi S, Hammel P, Hackert T, Jackson R, Büchler MW; European Study Group for Pancreatic Cancer. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. *Lancet* 2017; **389**: 1011-1024 [PMID: 28129987 DOI:

10.1016/S0140-6736(16)32409-6]
12 **Sinn M**, Bahra M, Liersch T, Gellert K, Messmann H, Bechstein W, Waldschmidt D, Jacobasch L, Wilhelm M, Rau BM, Grützmann R, Weinmann A, Maschmeyer G, Pelzer U, Stieler JM, Striefler JK, Ghadimi M, Bischoff S, Dörken B, Oettle H, Riess H. CONKO-005: Adjuvant Chemotherapy With Gemcitabine Plus Erlotinib Versus Gemcitabine Alone in Patients After R0 Resection of Pancreatic Cancer: A Multicenter Randomized Phase III Trial. *J Clin Oncol* 2017; **35**: 3330-3337 [PMID: 28817370 DOI: 10.1200/JCO.2017.72.6463]
13 **Uesaka K**, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, Kaneoka Y, Shimizu Y, Nakamori S, Sakamoto H, Morinaga S, Kainuma O, Imai K, Sata N, Hishinuma S, Ojima H, Yamaguchi R, Hirano S, Sudo T, Ohashi Y; JASPAC 01 Study Group. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). *Lancet* 2016; **388**: 248-257 [PMID: 27265347 DOI: 10.1016/S0140-6736(16)30583-9]
14 **Kindler HL**. A Glimmer of Hope for Pancreatic Cancer. *N Engl J Med* 2018; **379**: 2463-2464 [PMID: 30575492 DOI: 10.1056/NEJMe1813684]
15 **Rochefort MM**, Ankeny JS, Kadera BE, Donald GW, Isacoff W, Wainberg ZA, Hines OJ, Donahue TR, Reber HA, Tomlinson JS. Impact of tumor grade on pancreatic cancer prognosis: validation of a novel TNMG staging system. *Ann Surg Oncol* 2013; **20**: 4322-4329 [PMID: 23943022 DOI: 10.1245/s10434-013-3159-3]
16 **Javed MA**, Beyer G, Le N, Vinci A, Wong H, Palmer D, Morgan RD, Lamarca A, Hubner RA, Valle JW, Alam S, Chowdhury S, Ma YT, Archibugi L, Capurso G, Maisonneuve P, Neesse A, Sund M, Schober M, Krug S. Impact of intensified chemotherapy in metastatic pancreatic ductal adenocarcinoma (PDAC) in clinical routine in Europe. *Pancreatology* 2019; **19**: 97-104 [PMID: 30529068 DOI: 10.1016/j.pan.2018.10.003]
17 **Sharma MR**, Joshi SS, Karrison TG, Allen K, Suh G, Marsh R, Kozloff MF, Polite BN, Catenacci DVT, Kindler HL. A UGT1A1 genotype-guided dosing study of modified FOLFIRINOX in previously untreated patients with advanced gastrointestinal malignancies. *Cancer* 2019 [PMID: 30645764 DOI: 10.1002/cncr.31938]
18 **Shirasu H**, Todaka A, Omae K, Fujii H, Mizuno N, Ozaka M, Ueno H, Kobayashi S, Uesugi K, Kobayashi N, Hayashi H, Sudo K, Okano N, Horita Y, Kamei K, Yukisawa S, Kobayashi M, Fukutomi A. Impact of UGT1A1 genetic polymorphism on toxicity in unresectable pancreatic cancer patients undergoing FOLFIRINOX. *Cancer Sci* 2019; **110**: 707-716 [PMID: 30447099 DOI: 10.1111/cas.13883]
19 **Lee JM**, Lee HS, Hyun JJ, Choi HS, Kim ES, Keum B, Seo YS, Jeen YT, Chun HJ, Um SH, Kim CD. Prognostic value of inflammation-based markers in patients with pancreatic cancer administered gemcitabine and erlotinib. *World J Gastrointest Oncol* 2016; **8**: 555-562 [PMID: 27559435 DOI: 10.4251/wjgo.v8.i7.555]
20 **Shirai Y**, Shiba H, Sakamoto T, Horiuchi T, Haruki K, Fujiwara Y, Futagawa Y, Ohashi T, Yanaga K. Preoperative platelet to lymphocyte ratio predicts outcome of patients with pancreatic ductal adenocarcinoma after pancreatic resection. *Surgery* 2015; **158**: 360-365 [PMID: 26032829 DOI: 10.1016/j.surg.2015.03.043]
21 **Goldstein D**, El-Maraghi RH, Hammel P, Heinemann V, Kunzmann V, Sastre J, Scheithauer W, Siena S, Tabernero J, Teixeira L, Tortora G, Van Laethem JL, Young R, Penenberg DN, Lu B, Romano A, Von Hoff DD. nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. *J Natl Cancer Inst* 2015; 107 [PMID: 25638248 DOI: 10.1093/jnci/dju413]
22 **Luo G**, Guo M, Liu Z, Xiao Z, Jin K, Long J, Liu L, Liu C, Xu J, Ni Q, Yu X. Blood neutrophil-lymphocyte ratio predicts survival in patients with advanced pancreatic cancer treated with chemotherapy. *Ann Surg Oncol* 2015; **22**: 670-676 [PMID: 25155401 DOI: 10.1245/s10434-014-4021-y]
23 **Chen Y**, Yan H, Wang Y, Shi Y, Dai G. Significance of baseline and change in neutrophil-to-lymphocyte ratio in predicting prognosis: a retrospective analysis in advanced pancreatic ductal adenocarcinoma. *Sci Rep* 2017; **7**: 753 [PMID: 28392554 DOI: 10.1038/s41598-017-00859-5]
24 **Choi Y**, Oh DY, Park H, Kim TY, Lee KH, Han SW, Im SA, Kim TY, Bang YJ. More Accurate Prediction of Metastatic Pancreatic Cancer Patients' Survival with Prognostic Model Using Both Host Immunity and Tumor Metabolic Activity. *PLoS One* 2016; **11**: e0145692 [PMID: 26727470 DOI: 10.1371/journal.pone.0145692]
25 **de Jesus VHF**, Camandaroba MPG, Donadio MDS, Cabral A, Muniz TP, de Moura Leite L, Sant'Ana LF. Retrospective comparison of the efficacy and the toxicity of standard and modified FOLFIRINOX regimens in patients with metastatic pancreatic adenocarcinoma. *J Gastrointest Oncol* 2018; **9**: 694-707 [PMID: 30151266 DOI: 10.21037/jgo.2018.04.02]
26 **Yanagimoto H**, Satoi S, Sho M, Akahori T, Yamamoto T, Hirooka S, Yamaki S, Kotsuka M, Ryota H, Kinoshita S, Nishiwada S, Nagai M, Ikeda N, Tsuta K, Nakajima Y, Kon M. Phase I study assessing the feasibility of the triple combination chemotherapy of SOXIRI (S-1/oxaliplatin/irinotecan) in patients with unresectable pancreatic ductal adenocarcinoma. *Cancer Chemother Pharmacol* 2016; **77**: 35-41 [PMID: 26645403 DOI: 10.1007/s00280-015-2928-z]
27 **Akahori T**, Sho M, Yanagimoto H, Satoi S, Nagai M, Nishiwada S, Nakagawa K, Nakamura K, Yamamoto T, Hirooka S, Yamaki S, Ikeda N. Phase II Study of the Triple Combination Chemotherapy of SOXIRI (S-1/Oxaliplatin/Irinotecan) in Patients with Unresectable Pancreatic Ductal Adenocarcinoma. *Oncologist* 2019 [PMID: 30679316 DOI: 10.1634/theoncologist.2018-0900]
28 **Yang F**, Jin C, Jiang Y, Li J, Di Y, Ni Q, Fu D. Liposome based delivery systems in pancreatic cancer treatment: from bench to bedside. *Cancer Treat Rev* 2011; **37**: 633-642 [PMID: 21330062 DOI: 10.1016/j.ctrv.2011.01.006]
29 **Yang F**, Jin C, Subedi S, Lee CL, Wang Q, Jiang Y, Li J, Di Y, Fu D. Emerging inorganic nanomaterials for pancreatic cancer diagnosis and treatment. *Cancer Treat Rev* 2012; **38**: 566-579 [PMID: 22655679 DOI: 10.1016/j.ctrv.2012.02.003]
30 **Wang-Gillam A**, Li CP, Bodoky G, Dean A, Shan YS, Jameson G, Macarulla T, Lee KH, Cunningham D, Blanc JF, Hubner RA, Chiu CF, Schwartsmann G, Siveke JT, Braiteh F, Moyo V, Belanger B, Dhindsa N, Bayever E, Von Hoff DD, Chen LT; NAPOLI-1 Study Group. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. *Lancet* 2016; **387**: 545-557 [PMID: 26615328 DOI: 10.1016/S0140-6736(15)00986-1]
31 **Wang-Gillam A**, Hubner RA, Siveke JT, Von Hoff DD, Belanger B, de Jong FA, Mirakhur B, Chen LT. NAPOLI-1 phase 3 study of liposomal irinotecan in metastatic pancreatic cancer: Final overall survival analysis and characteristics of long-term survivors. *Eur J Cancer* 2019; **108**: 78-87 [PMID: 30654298

DOI: 10.1016/j.ejca.2018.12.007]
32 **Kang J**, Hwang I, Yoo C, Kim KP, Jeong JH, Chang HM, Lee SS, Park DH, Song TJ, Seo DW, Lee SK, Kim MH, Hong SM, Shin SH, Hwang DW, Song KB, Lee JH, Kim SC, Ryoo BY. Nab-paclitaxel plus gemcitabine versus FOLFIRINOX as the first-line chemotherapy for patients with metastatic pancreatic cancer: retrospective analysis. *Invest New Drugs* 2018; **36**: 732-741 [PMID: 29616439 DOI: 10.1007/s10637-018-0598-5]
33 **Michelakos T**, Pergolini I, Castillo CF, Honselmann KC, Cai L, Deshpande V, Wo JY, Ryan DP, Allen JN, Blaszkowsky LS, Clark JW, Murphy JE, Nipp RD, Parikh A, Qadan M, Warshaw AL, Hong TS, Lillemoe KD, Ferrone CR. Predictors of Resectability and Survival in Patients With Borderline and Locally Advanced Pancreatic Cancer who Underwent Neoadjuvant Treatment With FOLFIRINOX. *Ann Surg* 2019; **269**: 733-740 [PMID: 29227344 DOI: 10.1097/SLA.0000000000002600]
34 **Truty MJ**, Kendrick ML, Nagorney DM, Smoot RL, Cleary SP, Graham RP, Goenka AH, Hallemeier CL, Haddock MG, Harmsen WS, Mahipal A, McWilliams RR, Halfdanarson TR, Grothey AF. Factors Predicting Response, Perioperative Outcomes, and Survival Following Total Neoadjuvant Therapy for Borderline/Locally Advanced Pancreatic Cancer. *Ann Surg* 2019 [PMID: 30946090 DOI: 10.1097/SLA.0000000000003284]
35 **Casadei R**, Di Marco M, Ricci C, Santini D, Serra C, Calculli L, D'Ambra M, Guido A, Morselli-Labate AM, Minni F. Neoadjuvant Chemoradiotherapy and Surgery Versus Surgery Alone in Resectable Pancreatic Cancer: A Single-Center Prospective, Randomized, Controlled Trial Which Failed to Achieve Accrual Targets. *J Gastrointest Surg* 2015; **19**: 1802-1812 [PMID: 26224039 DOI: 10.1007/s11605-015-2890-4]
36 **Papalezova KT**, Tyler DS, Blazer DG, Clary BM, Czito BG, Hurwitz HI, Uronis HE, Pappas TN, Willett CG, White RR. Does preoperative therapy optimize outcomes in patients with resectable pancreatic cancer? *J Surg Oncol* 2012; **106**: 111-118 [PMID: 22311829 DOI: 10.1002/jso.23044]
37 **Motoi F**, Unno M, Takahashi H, Okada T, Wada K, Sho M, Nagano H, Matsumoto I, Satoi S, Murakami Y, Kishiwada M, Honda G, Kinoshita H, Baba H, Hishinuma S, Kitago M, Tajima H, Shinchi H, Takamori H, Kosuge T, Yamaue H, Takada T. Influence of preoperative anti-cancer therapy on resectability and perioperative outcomes in patients with pancreatic cancer: project study by the Japanese Society of Hepato-Biliary-Pancreatic Surgery. *J Hepatobiliary Pancreat Sci* 2014; **21**: 148-158 [PMID: 23913634 DOI: 10.1002/jhbp.15]
38 **Mokdad AA**, Minter RM, Zhu H, Augustine MM, Porembka MR, Wang SC, Yopp AC, Mansour JC, Choti MA, Polanco PM. Neoadjuvant Therapy Followed by Resection Versus Upfront Resection for Resectable Pancreatic Cancer: A Propensity Score Matched Analysis. *J Clin Oncol* 2017; **35**: 515-522 [PMID: 27621388 DOI: 10.1200/JCO.2016.68.5081]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2846-2862

DOI: 10.3748/wjg.v25.i23.2846

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Role of cytochrome P450 polymorphisms and functions in development of ulcerative colitis

Alaattin Sen, Holger Stark

ORCID number: Alaattin Sen (0000-0002-8444-376X); Holger Stark (0000-0003-3336-1710).

Author contributions: Sen A conceptualized and designed the review; Stark H drafted the initial manuscript; all authors reviewed and approved the final manuscript as submitted.

Conflict-of-interest statement: Authors declare no conflict of interest.

Manuscript source: Invited manuscript

Received: March 20, 2019
Peer-review started: March 20, 2019
First decision: April 5, 2019
Revised: April 26, 2019
Accepted: May 8, 2019
Article in press: May 8, 2019
Published online: June 21, 2019

P-Reviewer: Chiba T, Demonacos C
S-Editor: Ma RY
L-Editor: A

Alaattin Sen, Department of Molecular Biology and Genetics, Faculty of Life and Natural Sciences, Abdullah Gul University, Kayseri 38080, Turkey

Alaattin Sen, Biology Department, Faculty of Arts and Sciences, Pamukkale University, Denizli 20070, Turkey

Holger Stark, Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Duesseldorf 40225, Germany

Corresponding author: Alaattin Sen, PhD, Academic Fellow, Academic Research, Chairman, Full Professor, Instructor, Professor, Research Scientist, Senior Lecturer, Senior Researcher, Senior Scientist, Department of Molecular Biology and Genetics, Faculty of Life and Natural Sciences, Abdullah Gul University, Sumer Campus, Kocasinan, Kayseri 38080, Turkey.
sena@pau.edu.tr
Telephone: +90-352-2248800
Fax: +90-352-3388828

Abstract

Cytochromes P450s (CYPs) are terminal enzymes in CYP dependent monooxygenases, which constitute a superfamily of enzymes catalysing the metabolism of both endogenous and exogenous substances. One of their main tasks is to facilitate the excretion of these substances and eliminate their toxicities in most phase 1 reactions. Endogenous substrates of CYPs include steroids, bile acids, eicosanoids, cholesterol, vitamin D and neurotransmitters. About 80% of currently used drugs and environmental chemicals comprise exogenous substrates for CYPs. Genetic polymorphisms of CYPs may affect the enzyme functions and have been reported to be associated with various diseases and adverse drug reactions among different populations. In this review, we discuss the role of some critical CYP isoforms (CYP1A1, CYP2D6, CYP2J2, CYP2R1, CYP3A5, CYP3A7, CYP4F3, CYP24A1, CYP26B1 and CYP27B1) in the pathogenesis or aetiology of ulcerative colitis concerning gene polymorphisms. In addition, their significance in metabolism concerning ulcerative colitis in patients is also discussed showing a clear underestimation in genetic studies performed so far.

Key words: Cytochrome P450; Polymorphism; Function; Ulcerative colitis; Aetiology

Core tip: The role of cytochrome P450s (CYPs) genes in the pathogenesis of ulcerative

E-Editor: Ma YJ

colitis (UC). Extrahepatic and extrarenal CYPs (*e.g*., macrophages and dendritic cells of colonic mucosa) have a critical role in UC development. Polymorphisms discussed can result in dysregulation of these enzymes in favour of alternative pathways producing more reactive metabolites. Production of reactive metabolites is favouring more severe disease states. Pharmacogenetics might facilitate individualized medicine for UC in the future although actually available data is limited.

Citation: Sen A, Stark H. Role of cytochrome P450 polymorphisms and functions in development of ulcerative colitis. *World J Gastroenterol* 2019; 25(23): 2846-2862
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2846.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2846

INTRODUCTION

Cytochrome P450s (CYPs) are a superfamily of the integral membrane, heme-thiolate proteins entailed in the synthesis and breakdown of various molecules and chemicals within cells[1-3]. CYPs play a role in the metabolism of many endogenous substances including steroids, bile acids, eicosanoids, cholesterol, vitamin D and neurotransmitters, steroid hormones, cholesterol, fatty acids, bile acids[4-6]. Additional CYPs metabolise xenobiotics, such as drugs and endogenous molecules such as toxins that are shaped inside cells[7-11]. The human CYP isoenzymes superfamily is composed of 57 CYP genes and 58 pseudogenes arranged into 18 families and 43 subfamilies[12]. They are located either at endoplasmic reticulum or mitochondria of liver cells, but are also situated in other cells throughout the body[13-16]. Mitochondrial CYPs are commonly engaged in phase I reactions with anabolic and catabolic transformations of endogenous substances, while CYPs in the endoplasmic reticulum generally process xenobiotics. CYPs are gathered into families and subfamilies as indicated by the similarity index of the amino sequence. Every CYP is given a number relating a particular family inside the gene group, a letter exemplifying the subfamily, and a number allocated to the distinct gene inside the subgroup, *e.g*., the CYP gene that is in family 1, subgroup A, gene 1, is written as *CYP1A1*[12,17-20].

CYPs show intra- or interethnic and intra- or interindividual genetic variations. These variations or polymorphisms in CYP genes can largely alter the function of the enzymes. We continue to learn about the properties of these enzymes in humans and their roles in different diseases. As with many other genes and proteins associated with a critical life function, specific polymorphisms or variability in these CYPs and, hence, the gene product will result in pathology and lead to a severe human disease[21-24].

Ulcerative colitis (UC) is an idiopathic chronic inflammation condition with multifactorial determinants[25]. Populace-based careful surveys have shown that the frequency of UC worldwide has expanded in recent years. As opposed to the developed communities of North America and Western Europe, where the prevalence of UC has levelled or even lessened, publications demonstrate that incidences have elevated in developing countries, for example, those in Latin America, Asia and Eastern Europe leaving an urgent medical need[26-28]. The progression of UC requires a hereditary predilection, dysregulated immune reactions and an environmental incites. Candidate genes comprise those that govern innate immunity and epithelial boundary function[29-32]. Consequently, the interplay among hereditary and environmental components will cast the gut epithelial-inborn immunity interface and lead to unique phenotypes in patients with Inflammatory Bowel Disease.

Several studies have demonstrated that the CYP gene polymorphisms have been associated with the susceptibility to UC, but this is, to the best of our knowledge the first systematic review on the role of the CYPs polymorphism and function in the vulnerability and the development of UC. Each CYP will be described and discussed in alphabetical order, rather than in its importance to UC.

CYP1A1

CYP1A1 (EC 1.14.14.1), a notable aryl hydrocarbon hydroxylase, is expressed in the liver at exceptionally low quantities, and is mostly translated in human extrahepatic tissues, including digestive tract[8,33-37]. In humans, since the CYP1A1 is an extrahepatic

protein, it is considered to assume a secondary function in the removal of medications *in vivo*, and consequently, the polymorphism of the CYP1A1 gene may have a minor impact on their metabolic clearance. CYP1A1 assume essential tasks in the bioactivation of a collection of cancer-causing polycyclic aromatic hydrocarbons [PAHs; *e.g.*, benzo[a]pyrene (B[a]P)], aromatic amines and amides, and mycotoxins ascertained in certain grains, *e.g.*, aflatoxin B1[10,38-41]. CYP1A1 is also committed in the metabolism of endogenous compounds such as the pineal hormone melatonin and 17 β-estradiol and estrone[42-44]. It has been acknowledged as the principal CYP responsible for the C2- or C4-hydroxylation of 17 β-estradiol and estrone in extrahepatic tissues[45].

The CYP1A1 is positioned on chromosome 15 close to the mannose phosphate isomerase (MPI) locus at 15q22-24[46]. To date, there are 2092 SNPs depicted for CYP1A1 in NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp, access date: February 23, 2019). There are just a couple of investigations on the relationship of CYP1A1 polymorphism and function with UC.

CYP1A1 is the least studied isoform on the genotyping and phenotyping in gastroenterological practice, on the assumption that it is not involved in the metabolism of the intestine and inadequacy of clinical response. Klotz and colleagues[47] have tested the hypothesis that the appearance, arrangement, and activity of drug-metabolising enzymes in the gut may produce one or more reactive metabolites and create UC. For this purpose, they have evaluated the terminal ileum and different regions of the colon biopsies from 37 patients with UC by staining immunohistochemically for CYP1A1 isozyme. All proteins aside from CYP1A1 were displayed with comparable recurrence in both control and UC patients. CYP1A1 staining was definite substantially more frequently in patients with UC (39.4%) than in control (irritable colon, no clinical or histological indications of inflammations; 19.2%). These results may confirm the involvement of this protein in the aetiology or pathogenesis of inflammation in this tissue.

On the other hand, the more frequent appearance of CYP1A1 could be due to secondarily to the appearance of inflammation. Other recent studies have further supported the latter suggestion by demonstrating that the CYP1A1 regulates immune responses in the intestine and confers protection against intestinal inflammation[48-49]. On the other hand, Plewka and colleagues[50] have pointed out that the expression of CYP1A1 in enterocytes from UC patients was lower than in control, equivalent to 80% of the latter. Therefore, CYP1A1 needs to be further studied to resolve its role in UC.

Furthermore, our study included 161 Turkish patients with ulcerative colitis (94 males, 67 females; all Caucasian) consulting the outpatient clinic of the Department of Gastroenterology, Ege University, Turkey[51,52]. A group of 198 healthy Turkish Caucasians adjusted for age and sex (115 males and 83 females) were utilised as controls.

DNA preparations from the Turkish population were subjected to genotype analysis of CYP1A1*2B (rs4646903, T>C, 3′-flanking region, linked with increased enzyme activity). Our results showed that the CYP1A1*2A alleles correlate with an increased predisposition to UC, a piece of further supporting evidence that the increased CYP1A1 activity might cause the abundant accumulation of reactive metabolites, which advances an irregular intestinal immune response, causing irreversible harm to the colonic mucosa and eventually UC. Since there is limited and conflicting literature available on CYP1A1 polymorphism and function in UC, more studies are required to clarify the role of CYP1A1 in UC.

CYP2D6

The CYP2D6, also known as debrisoquine hydroxylase, presents a small percentage of all hepatic cytochrome P450s[53]. It is encoded by the CYP2D6 gene that is localised on chromosome 22q13.1 and participates in the biotransformation of about 20-25% of the clinically used drugs[54,55]. The endogenous substrates of CYP2D6 include neurotransmitters and neurosteroids, pinoline, progesterone and lipids[55,56]. It is highly polymorphic in the human population, and marked inter-racial variation observed.

Individuals are identified as ultra-rapid, extensive, intermediate or poor metabolizer, according to the number of functional alleles. To date, there are 3257 SNPs described for CYP2D6 in NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp, access date: February 23, 2019). CYP2D6 is another isoform that genotyping and phenotyping are not usually performed for gastroenterological inflammation, on the assumption that the irrelativity of the medications to intestinal digestion or generally little metabolic limit of CYP2D6 in the small digestive tract[57,58]. CYP2D6 is another isoform on which genotyping and phenotyping are not usually performed for

gastroenterological inflammation, on the belief that there is non-relativity of the medications to intestinal metabolism or the generally little metabolic limit of CYP2D6 in the small intestine[57,58].

Dudarewicz *et al*[59] have analysed CYP2D6 genotypes *1 (wild type), *3 and *4 using the PCR-RFLP method in 258 people from central Poland; 65 patients with UC and 50 with Crohn's disease (CD); and 143 healthy controls. They announced that despite the fact that the odds ratio (OR) was higher in carriers of the CYP2D6*1 /CYP2D6*3 genotype [extensive metabolizer (EM)], there was no factually significant difference in the recurrence of the CYP2D6 alleles in patients with UC and CD. Similarly, Trzcinski *et al*[60] have shown that the increased OR for inflammatory bowel disease (IBD) was without statistical significance. These researches suggest that there were no measurably significant variations in the appearance of the CYP2D6 alleles in IBD patients (UC or CD). However, the EM genotype might be the risk factor for IBD. Future investigations applied to a larger group of patients are needed to confirm presumptions.

Handersson *et al*[61] reported a real-world clinical case portraying the drug-drug interactions in a UC patient. The patient was on tamoxifen therapy for breast cancer and was prescribed rifampin for worsening UC. The patient is known to be a CYP2D6 intermediate metabolizer, and rifampin significantly lowered the endoxifen level. It constitutes a substantial risk of sub-therapeutic efficacy in tamoxifen patients, which may be of distinct concern among high-risk patients. This study illustrates the clinical value of CYPs genotyping in UC patients for therapeutic efficiencies of combinative therapies; exceptional consideration must be practised when required.

Le and Bae[62] carried out a meta-analysis probing the association between functional CYP2D6 polymorphisms (*3 and *4) and susceptibility to autoimmune diseases, including IBD. This meta-analysis demonstrated the association and susceptibility of the functional CYP2D6*3 and *4 polymorphisms with the autoimmune disease in Caucasians. Therefore, the CYP2D6 gene plays a role in the aetiology and the development of autoimmune diseases.

More direct evidence was very recently reported relating the CYP2D6*4 allele (PM) with susceptibility to UC[63]. The researchers studied CYP2D6*4 polymorphisms in 215 unrelated UC patients and 212 separate healthy controls by PCR-RFLP in a Kurdish population from Iran. A significantly higher frequency of CYP2D6*4 A allele in UC patients (12.6%) compared to healthy subjects (8.5%, $P = 0.046$) was reported. Also, the presence of A allele significantly increased the risk of UC by odds ratio (OR) = 1.56-fold ($P = 0.047$). This report is consistent with the Dudarewicz report showing a higher frequency of the CYP2D6*4 A allele in patients from Poland. Thus, this study suggests that CYP2D6*4 polymorphisms may be risk factors for UC susceptibility.

Moreover, a short while ago we reported the role of CYP2D6 in the metabolism of 5-aminosalicylic acid (5ASA), which is an anti-inflammatory drug used to treat ulcerative colitis. It is known that 5ASA is mainly metabolised to *N*-acetyl-5-ASA by *N*-acetyltransferases (NAT). However, no information is available on the oxidation of 5ASA by CYPs. Also, scarce pharmacogenetic analysis has focused directly on 5-ASA metabolism. Our study presented compelling evidence indicating that the 5-ASA is a substrate for CYP2D6[64,65]. Therefore, knowledge of CYP2D6 allelic variants is required for the better response of UC patients to this specific medication.

All these studies have strongly suggested that further studies are required to clarify the role of the CYP2D6 gene in aetiology, development and pathogenesis and the treatment of UC.

CYP2J2

CYP2J2 is the sole member of human CYP2J subfamily and the prominent arachidonic acid (AA) epoxygenases. It is localised on chromosome 1q32.1 and participates in the metabolism of AA to all four *cis*-eicosatrienoic acid epoxides (EETs) as 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, some of them exhibits signalling properties in anti-inflammatory pathways[66]. It is shown mainly in the cardiovascular system, yet is also displayed in the intestines, stomach, and other tissues[67-70].

Bystrom *et al*[71] demonstrated that CYP2J2 is an inflammation-induced enzyme displaying anti-inflammatory activities and raises phagocytosis of both gram-positive and negative bacteria. It was noted that the deficiency of CYP2J2 in IBD (CD) macrophages in response to bacterial infection may participate in the pathogenesis of the disease. Boosting the epoxygenase metabolites or the use of 11,12-EET mimetics may present useful therapeutic approaches for the treatment of IBD[66,71]. Therefore, the proper function of CYP2J2 is vital for bacterial clearance in IBD, and a deficit in the CYP2J2 pathway may advance bacterial pathogenesis resulting in the development of

IBD. Likewise, Qiu *et al*[72] assessed the EETs and the expression of CYP2J2 in colon tissue biopsies collected from UC patients along with adjacent unaffected tissues to study the role of CYP2J2 in UC. It was found that the quantities of EETs were significantly higher in UC tissues matched with adjacent unaffected tissues (1.91 ± 0.98 ng/mg *vs* 0.96 ± 0.77 ng/mg, mean ± SD, $P < 0.01$). Also, the expression of CYP2J2 rose significantly in UC tissues ($P < 0.05$). Thus, the increment in EET levels may be part of a defence mechanism in UC and CYP2J2 could be a key target for drug therapy for UC.

In addition, it was reported that endocannabinoids such as arachidonoyl ethanolamide (AEA) are significantly increased during intestinal inflammation and the CYP2J2 produced metabolites are related to pathologic conditions of the gastrointestinal tract[66,73-76]. CYP2J2 converts EAE to 20-hydroxyeicosatetraenoic acid ethanolamide (20-HETE-EA) and some other EET-EA metabolites which bind to cannabinoid-1 receptors (CB1-Rs) on neurons and cannabinoid-2 receptors (CB-2Rs) on immune and epithelial cells of the gut reducing digestion and represses the liberation of inflammatory mediators[77,78]. Also, UC patients display raised histamine levels which increase pathogenic neutrophil invasion into the colonic mucosa, intensifying the symptoms of colitis[79-82]. CYP2J2 has appeared to assume a fundamental role in the intestinal metabolism of antihistamines such as astemizole and ebastine. Thus, the knowledge of pharmacogenetics of CYP2J2 is essential and contribute to useful therapeutic approaches for the treatment of IBD.

There have been 7214 SNPs in human CYP2J2 gene in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 24 February 2019). A portion of these SNPs has been appeared to have a potential association with certain diseases, particularly cancer and heart diseases[83]. However, there is only a single report in the literature focusing on the relationship of CYP2J2 variation to UC. Otte and collaborators[84] have screened the polymorphism at position -50 (G-50T) in the promoter region of CYP2J2 (CYP2J2*7) in 146 UC and 147 CD patients matched to 357 healthy German people. CYP2J2*7 has a G>T replacement in the regulatory region at -50 position at the transcriptional start, causing diminished translation because of the loss of the Sp1 binding site[85,86]. Thus, this creates a smaller amounts of the CYP2J2 protein causing decreased CYP2J2 epoxygenase metabolites *in vivo*. The -50T allele was identified in 19.9% of subjects with UC 14.3% of subjects with CD, and 10.9% of the control group ($P < 0.05$). Additionally, a noteworthy higher recurrence of this allele was distinguished in patients with UC in contrast to the CD group. Their outcomes unequivocally support the relationship of UC with the promoter polymorphism in the CYP2J2 gene showing a critical function of epoxyeicosatrienoic acids in the pathophysiology of IBD. Further examinations are expected to depict the actions of CYP2J2 in pathology and the treatment of UC.

CYP2R1

The CYP2R1 gene produces an enzyme called vitamin D-25-hydroxylase (EC 1.14.14.24), showing a 25-hydroxylase action on both types of vitamin D, vitamin D2 and D3. It catalyses the initial reaction leading to the production of 1,25-dihydroxy vitamin D3, also called calcitriol[87]. CYP2R1 is located on chromosome 11p15.2 and converts vitamin D into 25-hydroxyvitamin D (calcidiol), which is the essential circulatory form of vitamin D.

There have been 4247 SNPs in human CYP2R1 gene in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 25 February 2019). However, there is no one study on the association of any of these SNPs with IBD, both CD and UC. Moreover, most studies focused on its role in vitamin D and related clinically significant diseases such as vitamin D deficiency[88]. However, few studies have examined its role in some autoimmune diseases such as multiple sclerosis, T1D, Hashimoto's disease and Grave's disease[89-94].

There is a convincing amount of evidence that vitamin D deficiency is one of a designated set of factors proposed to intervene in the contemplated relationship between environmental exposures and IBD, CD and UC[95-98]. It would be expected that the enzyme responsible for the production of active vitamin D, *i.e.*, CYP2R1, has some role in IBD. However, since it has been proposed that there is redundancy in the enzymes responsible for the 25-hydroxylation step, the identification of affected individuals with CYP2R1 polymorphisms has been difficult.

The rs10741657, located near the CYP2R1 gene has been linked by several studies to the increased risk of vitamin D insufficiency[99]. Moreover, an immense connection between the CYP2R1 gene transcript and 1,25(OH)2D3 plasma levels in the helper T cells was affirmed[99]. Ramos-Lopes *et al*[89] genotyped 203 simplex type 1 German

diabetes families for the rs10741657 polymorphism. They examined the 25(OH)D3 quantities comparing to CYP2R1 polymorphisms to its mRNA transcripts from peripheral blood mononuclear cells (PBMCs) in 133 T1D patients. The G variant of the rs10741657 polymorphism was more regularly transmitted to influenced children and was likewise more prevalent in cases than in the controls (46.1% *vs* 35.7%, $P = 0.03$). Patients conveying the genotype "GG" or "GA" of the rs10741657 polymorphism had, by and large, lower amounts of 25(OH)D3 in contrast to those with the genotype "AA". They showed an association of CYP2R1 polymorphisms with 25(OH)D3 levels in T1D patients. Coper *et al*[90] and Hussein *et al*[91] likewise detailed a similar association. Also, the relationship between the variant CYP2R1 alleles and MS risk suggested being dependency on the presence of the HLA-DR15 risk allele and other factors such as gender[89,94].

In conclusion, taking all of these studies into account, we have to remain sceptical regarding any association between CYP2R1 and IBD, both CD and UC, and emphasise that research efforts must be accelerated in oder to generate the answers. It would be not unexpected that CYP2R1 had a role on which we can only speculate with given data so far.

CYP3A4/3A5/3A7*

CYP3A4 (EC 1.14.13.97) is one of the most widely studied enzymes among CYPs since it metabolises approximately 60% of prescribed drugs, is localised mainly in the liver and in the intestine and is induced by glucocorticoids and some pharmacological agents[53,100]. In grown-ups, they are the main CYP3A subfamily members expressed in the liver and the intestine. CYP3A4 is the portion of a cluster of CYP genes on chromosome 7q22.1. It is called niphedipine oxidase but has many other aliases for its activity such as cholesterol 25-hydroxylase, taurochenodeoxycholate 6-alpha-hydroxylase and 1,8-cineole 2-exo-monooxygenase[101-103]. CYP3A4 also metabolises arachidonic acid to EETs or 20-HETE, expressing both epoxygenase and mono-oxygenase activities[104,105].

On the other hand, CYP3A5, a highly polymorphic enzyme, has been reported to range from undetectable amounts to amounts comparable to those of CYP3A4 in the human liver[106]. It is also part of a cluster of CYP genes on chromosome 7q21.1 and exhibits similar enzymatic activities[54,107].

5521 and 6608 SNPs have been found in human CYP3A4 and CYP3A5 genes, respectively in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 26 February 2019). However, there is no study on the association of any of these SNPs with IBD, both CD and UC. Yet, the role of variants in the transformation of the drugs used for the treatment of UC was studied in detail, particularly tacrolimus[108-110].

Tacrolimus is an immunosuppressive drug, utilised in the treatment of UC patients who have no response to 5-ASA or corticosteroids[111]. Tacrolimus is a substrate of CYP3A4 and CYP3A5, and they are in charge of the metabolism of tacrolimus[112]. CYP3A5's ancestral polymorphisms influence tacrolimus metabolism, for the most part. An SNP in the CYP3A5 gene including an A>G change at position 6986 inside intron 3 (rs776746) was determined to be strongly associated with CYP3A5 protein synthesis. CYP3A5*3/*3 genotypes are viewed as CYP3A5 non-expressers, while CYP3A5 expressers convey at least one CYP3A5*1 allele[113,114].

Many studies have shown that tacrolimus dose requirements are influenced by CYP3A4/5 genetic polymorphisms and the adverse events especially nephrotoxicity were frequently observed in CYP3A4/5 expressers. In addition, CYP3A4/5 expressers require that particular attention should be paid to the onset of nephrotoxicity. Thus, genotyping for CYP3A5 variants allows individualised care to be practised[57,108,110,115,116].

Plewka *et al*[50] reported that the CYP3A4 level was slightly higher in UC as compared to the control level. Additionally, in this case, the expression of CYP3A4 was restrained particularly in epithelial cells of the mucosa in the colon. The expression level of the fetal form of CYP3A, *i.e*., CYP3A7, was increased 3-fold in the colonic tissues of UC patients[117]. In addition, it was reported that CYP3A4 were induced by mesalazine (5-ASA), a drug used to treat colitis, in a concentration-dependent manner, both in cultured hepatocytes and human cryopreserved hepatocyte from UC[118]. Similarly, we have also determined that 5-ASA is both inducer and substrates for CYP3A4[64,65]. Accordingly, alteration in CYP3A4 activity in disease states may be an underappreciated determinant of difference in the aetiology of UC.

CYP4F3

CYP4F3 known as leukotriene-B4 omega-hydroxylase encodes two distinct enzymes and is part of a cluster of CYP genes on chromosome 19. CYP4F3 encodes two splice-variants, CYP4F3A and CYP4F3B, and their expression is tissue-specific with CYP3F3A being expressed mostly in leukocytes and CYP4F3B chiefly in the liver[119,120]. They metabolise leukotriene B4 and very likely 5-hydroxyeicosatetraenoic acid by an omega oxidation reaction, leading to the inactivation and degradation of well-known mediators of inflammation[121]. Thus, CYP4F has underlain the proposed roles of cytochromes in depressing inflammatory responses, and CYP4F3 is associated with IBD[122,123]. CYP4F3A/B also omega oxidise arachidonic acid to 20-HETE as well as EETs[124].

Five thousand five hundred and seventy-five SNPs have been found in human CYP4F3 in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 27 February 2019). Ananthakrishnan and colleagues[125] have genotyped CYP4F3 in 101 CD and 139 UC patients matched to 495 controls. They revealed that that high consumption of a high amount of n3:n6 PUFA (above median) exhibited a relationship to a decreased risk of UC (OR = 0.71, 95%CI: 0.47-1.09, P = 0.11). In addition, high n3:n6 PUFA intake was related with a decreased risk of UC in people with the GG/AG genotype (rs4646904) in CYP4F3 (OR = 0.57, 95%CI: 0.32-0.99) compared to those with the AA genotype (OR = 0.95, 95%CI: 0.47-1.93) (P-connection = 0.049). Despite the fact that the rs4646904 (converged into rs1805042) CYP4F3 variant is synonymous, it is proposed that it changes the splicing efficiency and gene expression level[126]. Hence, CYP4F3 variations are determinant of the relationship between dietary n3:n6 PUFA intake and the risk of UC. It is the only study present in the literature involving the CYP4F3 variants and an association between the UC. There is a definite need for additional studies examining the impact of other polymorphisms in exploring the association of specific CYP4F3 variants with UC as well as defining the diet-UC associations in patients.

CYP24A1

CYP24A1 is a mitochondrial monooxygenase which assumes a crucial function in calcium homeostasis through controlling the level of vitamin D3[127]. It is characterised as vitamin D3 24-hydroxylase (EC 1.14.15.16; Entrez Gene: CYP24A1 cytochrome P450 family 24 subfamily A part 1 [Homo sapiens (human)] accessed 28 February 2019). It facilitates hydroxylation reactions leading to the degradation of 1,25-dihydroxy vitamin D3, the physiologically active class of vitamin D. Hydroxylation of the side chain of vitamin D3 produces calcitroic acid and various metabolites which are discharged in bile. Inactivation of vitamin D is achieved by the mitochondrial catalyst, 25-hydroxyvitamin D3-24-hydroxylase, first portrayed in the mid-1970s and at first accepted to be included exclusively in the renal 24-hydroxylation of 25-OH-D3. Work performed over the ensuing 35 years has demonstrated that 24-hydroxylase is the aftereffect of CYP24A1[128,129]. CYP24A1 catalyses the transformation of both 25-OH-D3 and 1,25-(OH)2D3 into a series of 24- and 23-hydroxylated compounds directed to well-known pathways ending in the water-soluble biliary metabolite such as calcitroic acid and 26,23-lactone[130].

Besides being a self-induction of CYP24A1 by the 1,25-(OH)2D3 itself, the enzyme is controlled by crucial determinants such as the parathyroid hormone (PTH) and the fibroblast growth factor (FGF). 1,25-(OH)2D3-mediated PTH significantly reduces induction of the CYP24A1 expression because of destabilisation and enhanced degradation of CYP24A1 mRNA[130-132]. The translation of CYP24A1 is enhanced via PI3K-Akt-facilitated IRES within 5'UTR-dependent manner in response to the inflammatory condition by shifting from monosomal to polysomal fractions[133]. CYP24A1 is located on chromosome 20q13.2, and 6746 SNPs have been found in human CYP24A1 in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 28 February 2019).

CYP24A1 is correlated with idiopathic infantile hypercalcemia and is associated with the risk of cell-mediated immune mechanisms in MS and IBD[98,134,135]. The increased expression of CYP24A1 accompanied by CYP27B1 in inflamed colonic tissues in IBD patients results in the loss of 25(OH)D thereby exerting downward pressure on the vitamin D status. Therefore, it is essential in the regulation of inflammations.

Recently Chen *et al*[136] have genotyped rs4809957, rs6068816, rs6091822 and rs8124792 SNPs in 44 ulcerative colitis patients along with a control group composed of 504 East Asians enrolled in the 1000 Genomes Project. CYP24A1 polymorphisms rs4809957 A/G and rs6068816 C/T demonstrated a statistically noteworthy association with the UC when both the genotypes and allele frequencies were

considered together. Regarding rs6091822 G/T, UC was identified with risk allele bearers (GT + TT) *vs* wild-type (GG). However, the relationship between the allele frequencies and the disease were not significant. These alleles were additionally observed to be related to the risk of colonic polyps and colon cancer, and low-dose aspirin-related small intestine bleedings[137,138]. Rs4809957 situated in the 3′ untranslated vicinity neighbouring the polyA microsatellite repeat influences the stability of CYP24A1 mRNA and is proposed to be a coupling site for the retinoic acid-responsive element. Contrarily, rs4809957 may influence methylation of 3'UTR. It was accounted for that the 3'UTR of mRNA was the objective of miRNA to hasten degradation[139,140]. Rs6068816 likewise demonstrated a factually strong relationship with the risk of UC. Then OR for rs6068816 C *vs* T was again high (OR = 18.260, 95%CI: 8.350-39.932). Consequently, these discoveries demonstrate that rs6068816 T is a strong risk factor for UC as well. Changes in rs6068816 would not influence the amino acid residue of the CYP24A1 protein yet may influence intron splicing[141].

Interestingly, several SNPs in CYP24A1 and the other genes involved in vitamin D metabolism and signalling seem to exhibit susceptibility to UC in Asians, yet do not have a statistically significant effect on IBD risk in Europeans[136,142-145]. It is proposed that SNPs in CYP24A1 take part in the initiation or development of UC, and are not merely the result of ulcerative colitis-related malfunctions[136]. Despite the fact that the mechanisms are indistinct, it might be identified with vitamin D metabolism and signalling since both *in vivo* and in vitro investigations have shown the function of vitamin D in immune intervened diseases[98,145,146]. It is realised that vitamin D insufficiency cause diminished colonic bacterial clearance, decreased expression of tight junctions in the intestinal epithelium, and raised Th1 cell-driven inflammation at the gut level[97,127,145]. Therefore, CYPs in vitamin D metabolism are associated with the UC and deserve further detailed examinations.

CYP26B1

CYP26B1 is defined on chromosome 2p13.2, and the protein product is localised on the endoplasmic reticulum. It works as a crucial switch of all-*trans* retinoic acid (RA) levels. It inactivates all-trans retinoic acid to hydroxylated forms, including 4-OH-RA, 4-oxo-RA, and 18-OH-RA[147]. Mutations in this gene are related to radiohumeral fusions and other skeletal and craniofacial abnormalities, and an increased level of the protein is associated with atherosclerotic lesions. Alternative splicing results in multiple transcripts.

Kang *et al*[148] demonstrated that both high and low vitamin A levels brought about ameliorated intestinal inflammation and differentially activated subsets of FoxP3+ cells in SAMP1/YP mice. Likewise, Takeuchi *et al*[149] concluded that CYP26B1 expression was stimulated by all trans-RA in T-cells of the mesenteric lymph nodes (MLN) and Peyer's patches (PP) and changed the expression of CYP26B1 altering T cell dealing and separation in the gut. As of lately, Chenery *et al*[150] have demonstrated that CYP26B1 can restrict the differentiation of iTreg and Th17 cells and is differentially expressed by these cells to tweak RA responsiveness. In this manner, CYP26B1 in T cells is associated with the pathogenesis of T cell-mediated chronic inflammation in the colon, possibly by controlling T cell effector activity in the intestinal tissue.

Five thousand three hundred and thirty-nine SNPs have been found in human CYP26B1 in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 28 February 2019). There is only one study reporting the SNPs in CYP26B1 and the risk of IBD (UC and CD). Fransen *et al*[151] investigated the association of rs2241057 polymorphism with the threat of CD and UC, knowing that rs2241057 has an elevated catabolic function of retinoic acid. DNA from 1378 IBD patients (871 CD and 507 UC) and 1205 healthy controls gathered at Orebro University Hospital, and Karolinska University Hospital was genotyped for the CYP26B1 rs2241057 polymorphism. They detailed that a higher recurrence of patients homozygous for the dominant (T) allele was associated with the CD though not UC compared to the recurrence found in healthy controls. Hence, CYP26B1 polymorphism rs2241057 may have an increased risk for the progression of CD, which conceivably might be because of raised levels of RA. It was criticised that the lower number of enrolled patients with UC and the lack of standardisation in terms of ethnicity might be the reason for the absence of significant associations in UC. Therefore, their study requires further replication efforts and mechanistic studies to confirm the reported associations. In conclusion, further studies exploring vitamin A and CYP26B1 in the pathogenesis of both CD and UC are needed.

CYP27B1

The CYP27B1 gene supplies information to produce a mitochondrial protein called 25-dihydroxy vitamin D3 1-α-hydroxylase (EC 1.14.13.13). This catalyst performs the second of two consecutive reactions to transform vitamin D to its active structure, 1,25-dihydroxy vitamin D3 [1,25-(OH)2-D3], also called calcitriol. Vitamin D can be obtained from the diet or can be made on the body surface with the assistance of sunlight exposure. Whenever active, this vitamin is associated with retaining up the best possible balance of a few minerals in the body, including calcium and phosphate, which are fundamental for the regular composition of bones and teeth. Vitamin D is likewise engaged with a few other processes unrelated to bone and tooth organisation. It is currently kown that the protein exists in non-renal tissues to help increase the production of cellular 1,25-(OH)2-D3, a paracrine/autocrine system[129,152]. It affects immunomodulatory activities by repressing human leukocyte antigen (HLA) class II expression on endocrine cells, T cell proliferation and secretion of inflammatory cytokines that are thought to function in autoimmune tissue disruption[153-159]. In this way, the expression of CYP27B1 in cells of the colon, breast, prostate and monocyte/macrophage are so fundamental to the ordinary functioning of these tissues.

The cytogenetic location of the CYP27B1 is the long arm of chromosome 12 at position 14.1 (12q14.1). 1929 SNPs have been found in human CYP27B1 in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp, access date: 5 March 2019). Diseases associated with CYP27B1 include Type 1A and hypocalcemic vitamin D-dependent rickets and autoimmune diseases such as Addison, Hashimoto, Grave's, T1D and MS[158,160-164].

Du *et al*[159] surveyed the expressions of colonic CYP27B1 in UC patients. They gathered colon mucosal biopsies from the inflamed lesions and adjacent normal tissues from a cohort of patients with active UC. They revealed that CYP27B1 originating from colon epithelium, were notably induced in the lesions contrasting to the adjacent normal tissues in these patients. It was additionally bolstered by the observation that colon mucosal CYP27B1 was likewise induced significantly in an experimental colitis mouse model, and this nearby CYP27B1 induction and colonic inflammation required the community of commensal microscopic organisms. Therefore, in this context, it is vital to keep a regular vitamin D status with the goal that adequate substrate can be provided to CYP27B1, which produces local 1,25(OH)2D3 to protect the mucosal barrier and decrease colonic inflammation.

CONCLUSION

It is a widely accepted hypothesis that UC is caused by a reactive xenobiotic metabolite, which is conjugated before excretion. As was pointed out throughout this review, the function and the polymorphism of CYP1A1, 2D6, 2J2, 2R1, 3A4/5, 4F3, 24A1, 26B1 and 27B1 genes are undoubtedly important in the pathogenesis and clinical interest of UC, possibly producing reactive metabolites (Figure 1). These CYPs are somewhat involved in the metabolism of endogenous substrates, most notably vitamin D. It is evident that more of these polymorphisms are either loss-of-function mutations changing the amount of reactive metabolite produced and inhibiting or inducing the enzymes catalysing alternative pathways with the possibility of more severe conditions leading to disease states. However, some polymorphisms result in dysregulation of these enzymes leading to disease state. Most importantly, the exact role of the extrahepatic and extrarenal CYPs such as macrophages and dendritic cells of colonic mucosa is more critical to the development of UC and should require more thorough examinations to clarify their exact roles. The release of these metabolites resulted from polymorphic CYPs functions damage the colonic epithelial barrier and expose the mucosal immune system to luminal contents, thereby initiating an inflammatory response. It is undoubtedly an exciting moment to be involved in the study role of CYPs in autoimmune diseases like UC. An additional benefit is for rationalising the use of current therapeutics, *i.e*., administering the right drug at the right time and place to the right person. In addition to the polymorphism of CYPs, epigenetic regulation of UC cannot be excluded[165,166].

Early determination of disease-specific genes at the phase when their tolerance is still preserved would be the most critical since the essential treatment can be appropriately begun before auto-animosity happens. The candidate genes for genetic variant analysis was usually chosen from the genes associated with the pathogenesis of UC. Albeit a few SNPs have been identified to be related to drug response in patients with UC, most of these discoveries are as yet uncertain. Since not a single

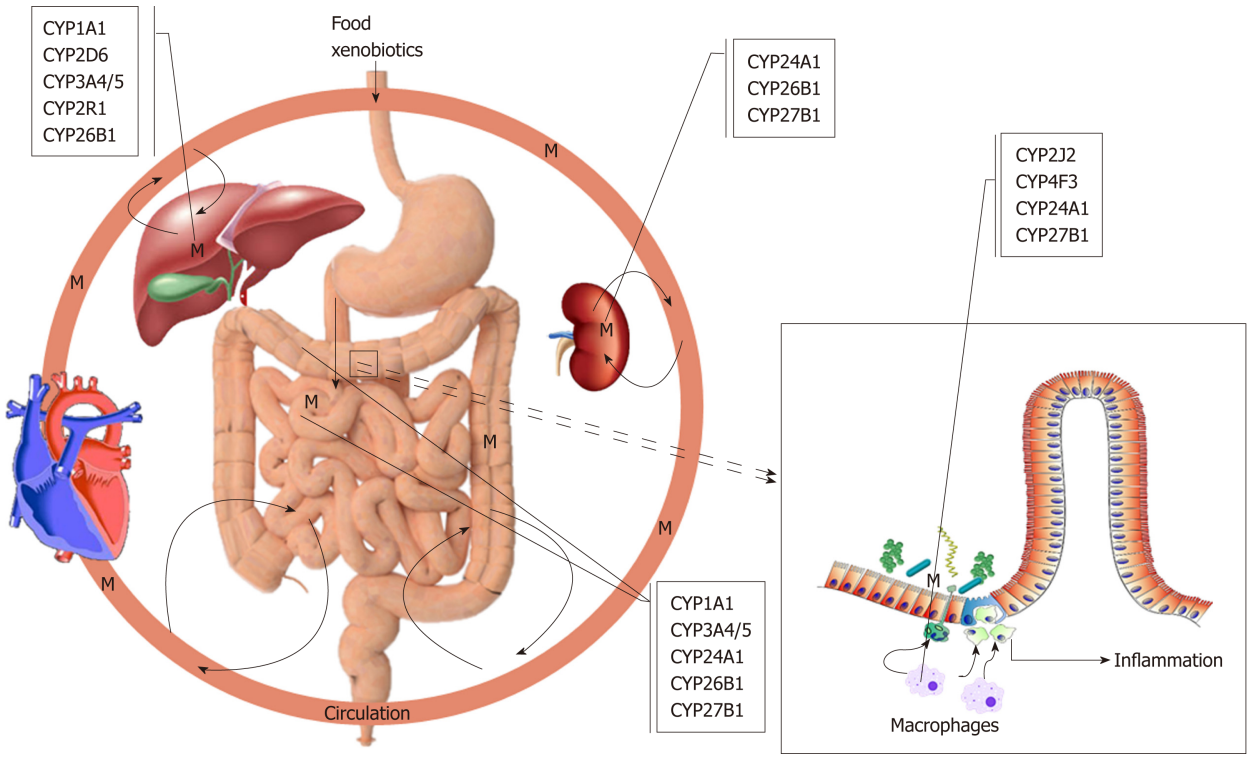


Figure 1 Overview the role of cytochromes P450s in ulcerative colitis. M: Metabolites; CYP: Cytochromes P450.

gene but rather different genes are engaged with the pathogenesis of UC just as drug responses are, a genome-wide association studies (GWAS) could be a progressively powerful methodology for distinguishing candidate genes to incorporate into these pharmacogenomic models. However, since the GWAS do not characterise the association between a gene and phenotype of the disease, discoveries from GWAS as well as the biological and clinical associations between the particular loci and diseases ought to be additionally investigated by conventional candidate gene examinations, for example, allelic separation by real-time PCR.

In future, more thorough investigations are necessary to elucidate the act of CYPs in the pathogenesis of UC. Furthermore, better learning of the role of genetic polymorphisms and haplotypes in CYPs expression and function will add to an excellent comprehension of inter-individual, ethnic and inter-ethnic variations in drug metabolism and impacts.

REFERENCES

1 **Omura T**, Sato R. A new cytochrome in liver microsomes. *J Biol Chem* 1962; **237**: 1375-1376 [PMID: 14482007]
2 **Nebert DW**, Russell DW. Clinical importance of the cytochromes P450. *Lancet* 2002; **360**: 1155-1162 [PMID: 12387968 DOI: 10.1016/S0140-6736(02)11203-7]
3 **Ortiz de Montellano PR**. Hydrocarbon hydroxylation by cytochrome P450 enzymes. *Chem Rev* 2010; **110**: 932-948 [PMID: 19769330 DOI: 10.1021/cr9002193]
4 **Williams RT**. Hepatic metabolism of drugs. *Gut* 1972; **13**: 579-585 [PMID: 4403576]
5 **Rendic S**, Guengerich FP. Survey of Human Oxidoreductases and Cytochrome P450 Enzymes Involved in the Metabolism of Xenobiotic and Natural Chemicals. *Chem Res Toxicol* 2015; **28**: 38-42 [PMID: 25485457 DOI: 10.1021/tx500444e]
6 **Guengerich FP**. Intersection of the Roles of Cytochrome P450 Enzymes with Xenobiotic and Endogenous Substrates: Relevance to Toxicity and Drug Interactions. *Chem Res Toxicol* 2017; **30**: 2-12 [PMID: 27472660 DOI: 10.1021/acs.chemrestox.6b00226]
7 **Yoshimoto FK**, Guengerich FP. Mechanism of the third oxidative step in the conversion of androgens to estrogens by cytochrome P450 19A1 steroid aromatase. *J Am Chem Soc* 2014; **136**: 15016-15025 [PMID: 25252141 DOI: 10.1021/ja508185d]
8 **Shimada T**, Yamazaki H, Mimura M, Wakamiya N, Ueng YF, Guengerich FP, Inui Y. Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. *Drug Metab Dispos* 1996; **24**: 515-522 [PMID: 8723730]
9 **Arinç E**, Sen A. In vivo effects of the anesthetic, benzocaine, on liver microsomal cytochrome P450 and mixed-function oxidase activities of gilthead seabream (Sparus aurata). *Comp Biochem Physiol Pharmacol Toxicol Endocrinol* 1994; **107**: 399-404 [PMID: 8061946]
10 **Sen A**, Arinç E. Further immunochemical and biocatalytic characterization of CYP1A1 from feral leaping mullet liver (Liza saliens) microsomes. *Comp Biochem Physiol C Toxicol Pharmacol* 2000; **126**: 235-244

[PMID: 11048673]
11 **Guengerich FP**, Waterman MR, Egli M. Recent Structural Insights into Cytochrome P450 Function. *Trends Pharmacol Sci* 2016; **37**: 625-640 [PMID: 27267697 DOI: 10.1016/j.tips.2016.05.006]
12 **Nelson DR**, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* 2004; **14**: 1-18 [PMID: 15128046]
13 **Porter TD**, Coon MJ. Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* 1991; **266**: 13469-13472 [PMID: 1856184]
14 **Thelen K**, Dressman JB. Cytochrome P450-mediated metabolism in the human gut wall. *J Pharm Pharmacol* 2009; **61**: 541-558 [PMID: 19405992 DOI: 10.1211/jpp/61.05.0002]
15 **Neve EP**, Ingelman-Sundberg M. Cytochrome P450 proteins: retention and distribution from the endoplasmic reticulum. *Curr Opin Drug Discov Devel* 2010; **13**: 78-85 [PMID: 20047148]
16 **Renaud HJ**, Cui JY, Khan M, Klaassen CD. Tissue distribution and gender-divergent expression of 78 cytochrome P450 mRNAs in mice. *Toxicol Sci* 2011; **124**: 261-277 [PMID: 21920951 DOI: 10.1093/toxsci/kfr240]
17 **Nebert DW**, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W. The P450 gene superfamily: recommended nomenclature. *DNA* 1987; **6**: 1-11 [PMID: 3829886 DOI: 10.1089/dna.1987.6.1]
18 **Elfaki I**, Mir R, Almutairi FM, Duhier FMA. Cytochrome P450: Polymorphisms and Roles in Cancer, Diabetes and Atherosclerosis. *Asian Pac J Cancer Prev* 2018; **19**: 2057-2070 [PMID: 30139042 DOI: 10.22034/APJCP.2018.19.8.2057]
19 **Sim SC**, Ingelman-Sundberg M. The Human Cytochrome P450 (CYP) Allele Nomenclature website: a peer-reviewed database of CYP variants and their associated effects. *Hum Genomics* 2010; **4**: 278-281 [PMID: 20511141]
20 **Sim SC**, Ingelman-Sundberg M. Update on allele nomenclature for human cytochromes P450 and the Human Cytochrome P450 Allele (CYP-allele) Nomenclature Database. *Methods Mol Biol* 2013; **987**: 251-259 [PMID: 23475683 DOI: 10.1007/978-1-62703-321-3_21]
21 **Makita K**, Falck JR, Capdevila JH. Cytochrome P450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system. *FASEB J* 1996; **10**: 1456-1463 [PMID: 8940291]
22 **Bell JC**, Strobel HW. Regulation of cytochrome P450 4F11 by nuclear transcription factor-κB. *Drug Metab Dispos* 2012; **40**: 205-211 [PMID: 22011441 DOI: 10.1124/dmd.111.041178]
23 **Nebert DW**, Wikvall K, Miller WL. Human cytochromes P450 in health and disease. *Philos Trans R Soc Lond B Biol Sci* 2013; **368**: 20120431 [PMID: 23297354 DOI: 10.1098/rstb.2012.0431]
24 **Pikuleva IA**, Waterman MR. Cytochromes p450: roles in diseases. *J Biol Chem* 2013; **288**: 17091-17098 [PMID: 23632021 DOI: 10.1074/jbc.R112.431916]
25 **Ungaro R**, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet* 2017; **389**: 1756-1770 [PMID: 27914657 DOI: 10.1016/S0140-6736(16)32126-2]
26 **Ng SC**, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 2018; **390**: 2769-2778 [PMID: 29050646 DOI: 10.1016/S0140-6736(17)32448-0]
27 **Sýkora J**, Pomahačová R, Kreslová M, Cvalínová D, Štych P, Schwarz J. Current global trends in the incidence of pediatric-onset inflammatory bowel disease. *World J Gastroenterol* 2018; **24**: 2741-2763 [PMID: 29991879 DOI: 10.3748/wjg.v24.i25.2741]
28 **da Silva BC**, Lyra AC, Rocha R, Santana GO. Epidemiology, demographic characteristics and prognostic predictors of ulcerative colitis. *World J Gastroenterol* 2014; **20**: 9458-9467 [PMID: 25071340 DOI: 10.3748/wjg.v20.i28.9458]
29 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185 DOI: 10.1038/nature06005]
30 **Abraham C**, Cho JH. Inflammatory bowel disease. *N Engl J Med* 2009; **361**: 2066-2078 [PMID: 19923578 DOI: 10.1056/NEJMra0804647]
31 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]
32 **Luo Y**, de Lange KM, Jostins L, Moutsianas L, Randall J, Kennedy NA, Lamb CA, McCarthy S, Ahmad T, Edwards C, Serra EG, Hart A, Hawkey C, Mansfield JC, Mowat C, Newman WG, Nichols S, Pollard M, Satsangi J, Simmons A, Tremelling M, Uhlig H, Wilson DC, Lee JC, Prescott NJ, Lees CW, Mathew CG, Parkes M, Barrett JC, Anderson CA. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. *Nat Genet* 2017; **49**: 186-192 [PMID: 28067910 DOI: 10.1038/ng.3761]
33 **Huang H**, Fang M, Jostins L, Umićević Mirkov M, Boucher G, Anderson CA, Andersen V, Cleynen I, Cortes A, Crins F, D'Amato M, Deffontaine V, Dmitrieva J, Docampo E, Elansary M, Farh KK, Franke A, Gori AS, Goyette P, Halfvarson J, Haritunians T, Knight J, Lawrance IC, Lees CW, Louis E, Mariman R, Meuwissen T, Mni M, Momozawa Y, Parkes M, Spain SL, Théâtre E, Trynka G, Satsangi J, van Sommeren S, Vermeire S, Xavier RJ; International Inflammatory Bowel Disease Genetics Consortium, Weersma RK, Duerr RH, Mathew CG, Rioux JD, McGovern DPB, Cho JH, Georges M, Daly MJ, Barrett JC. Fine-mapping inflammatory bowel disease loci to single-variant resolution. *Nature* 2017; **547**: 173-178 [PMID: 28658209 DOI: 10.1038/nature22969]
34 **Mhandire D**, Lacerda M, Castel S, Mhandire K, Zhou D, Swart M, Shamu T, Smith P, Musingwini T, Wiesner L, Stray-Pedersen B, Dandara C. Effects of CYP2B6 and CYP1A2 Genetic Variation on Nevirapine Plasma Concentration and Pharmacodynamics as Measured by CD4 Cell Count in Zimbabwean HIV-Infected Patients. *OMICS* 2015; **19**: 553-562 [PMID: 26348712 DOI: 10.1089/omi.2015.0104]
35 **Paine MF**, Schmiedlin-Ren P, Watkins PB. Cytochrome P-450 1A1 expression in human small bowel: interindividual variation and inhibition by ketoconazole. *Drug Metab Dispos* 1999; **27**: 360-364 [PMID: 10064566]
36 **Prueksaritanont T**, Gorham LM, Hochman JH, Tran LO, Vyas KP. Comparative studies of drug-metabolizing enzymes in dog, monkey, and human small intestines, and in Caco-2 cells. *Drug Metab Dispos* 1996; **24**: 634-642 [PMID: 8781778]
37 **Willey JC**, Coy EL, Frampton MW, Torres A, Apostolakos MJ, Hoehn G, Schuermann WH, Thilly WG, Olson DE, Hammersley JR, Crespi CL, Utell MJ. Quantitative RT-PCR measurement of cytochromes p450 1A1, 1B1, and 2B7, microsomal epoxide hydrolase, and NADPH oxidoreductase expression in lung

cells of smokers and nonsmokers. *Am J Respir Cell Mol Biol* 1997; **17**: 114-124 [PMID: 9224217 DOI: 10.1165/ajrcmb.17.1.2783]
38 **Dey A**, Parmar D, Dayal M, Dhawan A, Seth PK. Cytochrome P450 1A1 (CYP1A1) in blood lymphocytes evidence for catalytic activity and mRNA expression. *Life Sci* 2001; **69**: 383-393 [PMID: 11459429]
39 **Guengerich FP**, Shimada T. Activation of procarcinogens by human cytochrome P450 enzymes. *Mutat Res* 1998; **400**: 201-213 [PMID: 9685642]
40 **Oda Y**, Aryal P, Terashita T, Gillam EM, Guengerich FP, Shimada T. Metabolic activation of heterocyclic amines and other procarcinogens in Salmonella typhimurium umu tester strains expressing human cytochrome P4501A1, 1A2, 1B1, 2C9, 2D6, 2E1, and 3A4 and human NADPH-P450 reductase and bacterial O-acetyltransferase. *Mutat Res* 2001; **492**: 81-90 [PMID: 11377247]
41 **Eshkoor SA**, Ismail P, Rahman SA. Does CYP1A1 gene polymorphism affect cell damage biomarkers and ageing? *Turk J Biol* 2014; **38**: 219-225 [DOI: 10.3906/biy-1308-61]
42 **Spink DC**, Hayes CL, Young NR, Christou M, Sutter TR, Jefcoate CR, Gierthy JF. The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on estrogen metabolism in MCF-7 breast cancer cells: evidence for induction of a novel 17 beta-estradiol 4-hydroxylase. *J Steroid Biochem Mol Biol* 1994; **51**: 251-258 [PMID: 7826886]
43 **Schwarz D**, Kisselev P, Schunck WH, Chernogolov A, Boidol W, Cascorbi I, Roots I. Allelic variants of human cytochrome P450 1A1 (CYP1A1): effect of T461N and I462V substitutions on steroid hydroxylase specificity. *Pharmacogenetics* 2000; **10**: 519-530 [PMID: 10975606]
44 **Ma X**, Idle JR, Krausz KW, Gonzalez FJ. Metabolism of melatonin by human cytochromes p450. *Drug Metab Dispos* 2005; **33**: 489-494 [PMID: 15616152 DOI: 10.1124/dmd.104.002410]
45 **Lee AJ**, Cai MX, Thomas PE, Conney AH, Zhu BT. Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* 2003; **144**: 3382-3398 [PMID: 12865317 DOI: 10.1210/en.2003-0192]
46 **Hildebrand CE**, Gonzalez FJ, McBride OW, Nebert DW. Assignment of the human 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P1-450 gene to chromosome 15. *Nucleic Acids Res* 1985; **13**: 2009-2016 [PMID: 4000952]
47 **Klotz U**, Hoensch H, Schütz T, Beaune P, Zanger U, Bode JC, Fritz P. Expression of intestinal drug-metabolizing enzymes in patients with chronic inflammatory bowel disease. *Curr Ther Res* 1998; **59**: 556–563 [DOI: 10.1016/S0011-393X(98)85095-9]
48 **Arsenescu R**, Arsenescu V, Zhong J, Nasser M, Melinte R, Dingle RW, Swanson H, de Villiers WJ. Role of the xenobiotic receptor in inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 1149-1162 [PMID: 20878756 DOI: 10.1002/ibd.21463]
49 **Manzella C**, Singhal M, Alrefai WA, Saksena S, Dudeja PK, Gill RK. Serotonin is an endogenous regulator of intestinal CYP1A1 via AhR. *Sci Rep* 2018; **8**: 6103 [PMID: 29666456 DOI: 10.1038/s41598-018-24213-5]
50 **Plewka D**, Plewka A, Szczepanik T, Morek M, Bogunia E, Wittek P, Kijonka C. Expression of selected cytochrome P450 isoforms and of cooperating enzymes in colorectal tissues in selected pathological conditions. *Pathol Res Pract* 2014; **210**: 242-249 [PMID: 24485758 DOI: 10.1016/j.prp.2013.12.010]
51 **Ozkarsli M**, Buyukgoze O, Sabah S, Osmanoglu N, Sen A. Genetic polymorphisms of biotransformation enzymes CYP1A1 P4502A6 and microsomal epoxide hydrolase. *FEBS Journal* 2008; **275**: 110 [DOI: 10.1111/j.1742-4658.2008.06444.x]
52 **Buyukgoze O**, Osmanoglu N, Arslan S, Sen A. Association of the CYP1A1*2A, GSTT1 null, GSTM1 null, mEPHX*3, and XRCC1-399 genetic polymorphisms with ulcerative colitis. *Int J Colorectal Dis* 2013; **28**: 593-595 [PMID: 22664944 DOI: 10.1007/s00384-012-1507-6]
53 **Ingelman-Sundberg M**. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J* 2005; **5**: 6-13 [PMID: 15492763 DOI: 10.1038/sj.tpj.6500285]
54 **Givens RC**, Watkins PB. Pharmacogenetics and clinical gastroenterology. *Gastroenterology* 2003; **125**: 240-248 [DOI: 10.1016/S0016-5085(03)00683-8]
55 **Teh LK**, Bertilsson L. Pharmacogenomics of CYP2D6: molecular genetics, interethnic differences and clinical importance. *Drug Metab Pharmacokinet* 2012; **27**: 55-67 [PMID: 22185816]
56 **Wang X**, Li J, Dong G, Yue J. The endogenous substrates of brain CYP2D. *Eur J Pharmacol* 2014; **724**: 211-218 [PMID: 24374199 DOI: 10.1016/j.ejphar.2013.12.025]
57 **Camilleri M**. The role of pharmacogenetics in nonmalignant gastrointestinal diseases. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 173-184 [PMID: 22310916 DOI: 10.1038/nrgastro.2012.2]
58 **Kawakami M**, Takenoshita-Nakaya S, Takeba Y, Nishimura Y, Oda M, Watanabe M, Ohta Y, Kobayashi S, Ohtsubo T, Kobayashi S, Uchida N, Matsumoto N. Evaluation of CYP2D6 Protein Expression and Activity in the Small Intestine to Determine Its Metabolic Capability in the Japanese Population. *Biol Pharm Bull* 2017; **40**: 1344-1351 [PMID: 28626197 DOI: 10.1248/bpb.b16-00370]
59 **Dudarewicz M**, Rychlik-Sych M, Barańska M, Wojtczak A, Trzciński R, Dziki A, Skrętkowicz J. Significance of the genetic polymorphism of CYP2D6 and NAT2 in patients with inflammatory bowel diseases. *Pharmacol Rep* 2014; **66**: 686-690 [PMID: 24948072 DOI: 10.1016/j.pharep.2014.04.002]
60 **Trzcinski R**, Skretkowicz J, Dziki A, Rychlik-Sych M, Baranska M. Genetic polymorphisms of CYP2D6 oxidation in patients with inflammatory bowel disease. *Dig Dis Sci* 2010; **55**: 1037-1043 [PMID: 19437119 DOI: 10.1007/s10620-009-0816-2]
61 **Henderson SL**, Teft WA, Kim RB. Profound reduction in tamoxifen active metabolite endoxifen in a breast cancer patient treated with rifampin prior to initiation of an anti-TNFα biologic for ulcerative colitis: a case report. *BMC Cancer* 2016; **16**: 304 [PMID: 27169677 DOI: 10.1186/s12885-016-2342-x]
62 **Lee YH**, Bae SC. Association between Functional CYP2D6 Polymorphisms and Susceptibility to Autoimmune Diseases: A Meta-Analysis. *Immunol Invest* 2017; **46**: 109-122 [PMID: 27749127 DOI: 10.1080/08820139.2016.1226898]
63 **Lotfi F**, Bahrehmand F, Vaisi-Raygani A, Khodarahmi R, Tanhapour M, Kiani A, Rahimi Z, Pourmotabbed T. Cytochrome P450 (CYP450,2D6*A), N-Acetyltransferase-2 (NAT2*7, A) and Multidrug Resistance 1 (MDR1 3435 T) Alleles Collectively Increase Risk of Ulcerative Colitis. *Arch Iran Med* 2018; **21**: 530-535 [PMID: 30551694]
64 **Kale E**, Sen A. Identification of the cytochrome P4502D6 in the metabolism of 5-aminosalicylic acid: in vitro investigations of potential co-prescription interactions. The 3rd International Symposium on EuroAsian Biodiversity, Minsk, Belarus, 5-8 July, 2017. Abstract Book 484-484. Available from: http://elib.bsu.by/bitstream/123456789/180188/1/p200.pdf
65 **Kale E**, Sen A. Characterization of Human CYP450 Isozymes Responsible For The In Vitro Oxidative

Metabolism of Mesalamine Used For Colitis. *Innovations in Science and Education* 2019; **7**: In Press
66 **Walker VJ**, Griffin AP, Hammar DK, Hollenberg PF. Metabolism of Anandamide by Human Cytochrome P450 2J2 in the Reconstituted System and Human Intestinal Microsomes. *J Pharmacol Exp Ther* 2016; **357**: 537-544 [PMID: 27000802 DOI: 10.1124/jpet.116.232553]
67 **Wu S**, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J Biol Chem* 1996; **271**: 3460-3468 [PMID: 8631948]
68 **Enayetallah AE**, French RA, Thibodeau MS, Grant DF. Distribution of soluble epoxide hydrolase and of cytochrome P450 2C8, 2C9, and 2J2 in human tissues. *J Histochem Cytochem* 2004; **52**: 447-454 [PMID: 15033996 DOI: 10.1177/002215540405200403]
69 **Paine MF**, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos* 2006; **34**: 880-886 [PMID: 16467132 DOI: 10.1124/dmd.105.008672]
70 **Karkhanis A**, Hong Y, Chan ECY. Inhibition and inactivation of human CYP2J2: Implications in cardiac pathophysiology and opportunities in cancer therapy. *Biochem Pharmacol* 2017; **135**: 12-21 [PMID: 28237650 DOI: 10.1016/j.bcp.2017.02.017]
71 **Bystrom J**, Thomson SJ, Johansson J, Edin ML, Zeldin DC, Gilroy DW, Smith AM, Bishop-Bailey D. Inducible CYP2J2 and its product 11,12-EET promotes bacterial phagocytosis: a role for CYP2J2 deficiency in the pathogenesis of Crohn's disease? *PLoS One* 2013; **8**: e75107 [PMID: 24058654 DOI: 10.1371/journal.pone.0075107]
72 **Qiu YE**, Qin J, Luo Y, Qin SL, Mu YF, Cun R, Jiang HL, Chen JJ, Yu MH, Zhong M. Increased epoxyeicosatrienoic acids may be part of a protective mechanism in human ulcerative colitis, with increased CYP2J2 and reduced soluble epoxide hydrolase expression. *Prostaglandins Other Lipid Mediat* 2018; **136**: 9-14 [PMID: 29580941 DOI: 10.1016/j.prostaglandins.2018.03.004]
73 **Izzo AA**, Fezza F, Capasso R, Bisogno T, Pinto L, Iuvone T, Esposito G, Mascolo N, Di Marzo V, Capasso F. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001; **134**: 563-570 [PMID: 11588110 DOI: 10.1038/sj.bjp.0704293]
74 **D'Argenio G**, Valenti M, Scaglione G, Cosenza V, Sorrentini I, Di Marzo V. Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB J* 2006; **20**: 568-570 [PMID: 16403786 DOI: 10.1096/fj.05-4943fje]
75 **Di Marzo V**, Izzo AA. Endocannabinoid overactivity and intestinal inflammation. *Gut* 2006; **55**: 1373-1376 [PMID: 16966693 DOI: 10.1136/gut.2005.090472]
76 **Askari AA**, Thomson S, Edin ML, Lih FB, Zeldin DC, Bishop-Bailey D. Basal and inducible anti-inflammatory epoxygenase activity in endothelial cells. *Biochem Biophys Res Commun* 2014; **446**: 633-637 [PMID: 24631907 DOI: 10.1016/j.bbrc.2014.03.020]
77 **Izzo AA**. Cannabinoids and intestinal motility: welcome to CB2 receptors. *Br J Pharmacol* 2004; **142**: 1201-1202 [PMID: 15277313 DOI: 10.1038/sj.bjp.0705890]
78 **Mathison R**, Ho W, Pittman QJ, Davison JS, Sharkey KA. Effects of cannabinoid receptor-2 activation on accelerated gastrointestinal transit in lipopolysaccharide-treated rats. *Br J Pharmacol* 2004; **142**: 1247-1254 [PMID: 15249429 DOI: 10.1038/sj.bjp.0705889]
79 **Nolte H**, Spjeldnaes N, Kruse A, Windelborg B. Histamine release from gut mast cells from patients with inflammatory bowel diseases. *Gut* 1990; **31**: 791-794 [PMID: 1695160]
80 **King T**, Biddle W, Bhatia P, Moore J, Miner PB. Colonic mucosal mast cell distribution at line of demarcation of active ulcerative colitis. *Dig Dis Sci* 1992; **37**: 490-495 [PMID: 1551335]
81 **Lennon EM**, Borst LB, Edwards LL, Moeser AJ. Mast Cells Exert Anti-Inflammatory Effects in an IL10^{-/-} Model of Spontaneous Colitis. *Mediators Inflamm* 2018; **2018**: 7817360 [PMID: 29849494 DOI: 10.1155/2018/7817360]
82 **Wechsler JB**, Szabo A, Hsu CL, Krier-Burris RA, Schroeder HA, Wang MY, Carter RG, Velez TE, Aguiniga LM, Brown JB, Miller ML, Wershil BK, Barrett TA, Bryce PJ. Histamine drives severity of innate inflammation via histamine 4 receptor in murine experimental colitis. *Mucosal Immunol* 2018; **11**: 861-870 [PMID: 29363669 DOI: 10.1038/mi.2017.121]
83 **Xu M**, Ju W, Hao H, Wang G, Li P. Cytochrome P450 2J2: distribution, function, regulation, genetic polymorphisms and clinical significance. *Drug Metab Rev* 2013; **45**: 311-352 [PMID: 23865864 DOI: 10.3109/03602532.2013.806537]
84 **Otte JM**, Spiecker M, Jagiello P, Brand S, Felderbauer P, Griga T, Epplen JT, Schmitz F, Schmidt WE. Crohn's disease and ulcerative colitis are associated with a promoter polymorphism of the cytochrome P450 epoxygenase CYP2J2 gene. *Gastroenterology* 2005; **128**: A446-A447 [DOI: 10.1053/j.gastro.2005.04.003]
85 **King LM**, Ma J, Srettabunjong S, Graves J, Bradbury JA, Li L, Spiecker M, Liao JK, Mohrenweiser H, Zeldin DC. Cloning of CYP2J2 gene and identification of functional polymorphisms. *Mol Pharmacol* 2002; **61**: 840-852 [PMID: 11901223]
86 **Spiecker M**, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, Node K, Börgel J, Mügge A, Lindpaintner K, Huesing A, Maisch B, Zeldin DC, Liao JK. Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. *Circulation* 2004; **110**: 2132-2136 [PMID: 15466638 DOI: 10.1161/01.CIR.0000143832.91812.60]
87 **Cheng JB**, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci U S A* 2004; **101**: 7711-7715 [PMID: 15128933 DOI: 10.1073/pnas.0402490101]
88 **Slater NA**, Rager ML, Havrda DE, Harralson AF. Genetic Variation in CYP2R1 and GC Genes Associated With Vitamin D Deficiency Status. *J Pharm Pract* 2017; **30**: 31-36 [PMID: 26038244 DOI: 10.1177/0897190015585876]
89 **Ramos-Lopez E**, Brück P, Jansen T, Herwig J, Badenhoop K. CYP2R1 (vitamin D 25-hydroxylase) gene is associated with susceptibility to type 1 diabetes and vitamin D levels in Germans. *Diabetes Metab Res Rev* 2007; **23**: 631-636 [PMID: 17607662 DOI: 10.1002/dmrr.719]
90 **Cooper JD**, Smyth DJ, Walker NM, Stevens H, Burren OS, Wallace C, Greissl C, Ramos-Lopez E, Hyppönen E, Dunger DB, Spector TD, Ouwehand WH, Wang TJ, Badenhoop K, Todd JA. Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. *Diabetes* 2011; **60**: 1624-1631 [PMID: 21441443 DOI: 10.2337/db10-1656]
91 **Hussein AG**, Mohamed RH, Alghobashy AA. Synergism of CYP2R1 and CYP27B1 polymorphisms and susceptibility to type 1 diabetes in Egyptian children. *Cell Immunol* 2012; **279**: 42-45 [PMID: 23063903 DOI: 10.1016/j.cellimm.2012.08.006]

92 **Inoue N**, Watanabe M, Ishido N, Katsumata Y, Kagawa T, Hidaka Y, Iwatani Y. The functional polymorphisms of VDR, GC and CYP2R1 are involved in the pathogenesis of autoimmune thyroid diseases. *Clin Exp Immunol* 2014; **178**: 262-269 [PMID: 25046415 DOI: 10.1111/cei.12420]

93 **Al-Barry MA**, Albalawi AM, Sayf MA, Badawi A, Afzal S, Latif M, Samman MI, Basit S. Sequence analysis of four vitamin D family genes (VDR, CYP24A1, CYP27B1 and CYP2R1) in Vogt-Koyanagi-Harada (VKH) patients: identification of a potentially pathogenic variant in CYP2R1. *BMC Ophthalmol* 2016; **16**: 172 [PMID: 27716192 DOI: 10.1186/s12886-016-0354-6]

94 **Scazzone C**, Agnello L, Ragonese P, Lo Sasso B, Bellia C, Bivona G, Schillaci R, Salemi G, Ciaccio M. Association of CYP2R1 rs10766197 with MS risk and disease progression. *J Neurosci Res* 2018; **96**: 297-304 [PMID: 28834557 DOI: 10.1002/jnr.24133]

95 **El-Matary W**, Sikora S, Spady D. Bone mineral density, vitamin D, and disease activity in children newly diagnosed with inflammatory bowel disease. *Dig Dis Sci* 2011; **56**: 825-829 [PMID: 20725784 DOI: 10.1007/s10620-010-1380-5]

96 **Ulitsky A**, Ananthakrishnan AN, Naik A, Skaros S, Zadvornova Y, Binion DG, Issa M. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. *JPEN J Parenter Enteral Nutr* 2011; **35**: 308-316 [PMID: 21527593 DOI: 10.1177/0148607110381267]

97 **Palmer MT**, Weaver CT. Linking vitamin d deficiency to inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 2245-2256 [PMID: 23591600 DOI: 10.1097/MIB.0b013e31828a3b6f]

98 **Del Pinto R**, Pietropaoli D, Chandar AK, Ferri C, Cominelli F. Association Between Inflammatory Bowel Disease and Vitamin D Deficiency: A Systematic Review and Meta-analysis. *Inflamm Bowel Dis* 2015; **21**: 2708-2717 [PMID: 26348447 DOI: 10.1097/MIB.0000000000000546]

99 **Wang TJ**, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouhi NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasan RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Foroud T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Järvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet* 2010; **376**: 180-188 [PMID: 20541252 DOI: 10.1016/S0140-6736(10)60588-0]

100 **Morán-Auth Y**, Penna-Martinez M, Shoghi F, Ramos-Lopez E, Badenhoop K. Vitamin D status and gene transcription in immune cells. *J Steroid Biochem Mol Biol* 2013; **136**: 83-85 [PMID: 23416105 DOI: 10.1016/j.jsbmb.2013.02.005]

101 **Zanger UM**, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* 2013; **138**: 103-141 [PMID: 23333322 DOI: 10.1016/j.pharmthera.2012.12.007]

102 **Ashida R**, Okamura Y, Ohshima K, Kakuda Y, Uesaka K, Sugiura T, Ito T, Yamamoto Y, Sugino T, Urakami K, Kusuhara M, Yamaguchi K. CYP3A4 Gene Is a Novel Biomarker for Predicting a Poor Prognosis in Hepatocellular Carcinoma. *Cancer Genomics Proteomics* 2017; **14**: 445-453 [PMID: 29109094 DOI: 10.21873/cgp.20054]

103 **Gupta RP**, He YA, Patrick KS, Halpert JR, Bell NH. CYP3A4 is a vitamin D-24- and 25-hydroxylase: analysis of structure function by site-directed mutagenesis. *J Clin Endocrinol Metab* 2005; **90**: 1210-1219 [PMID: 15546903 DOI: 10.1210/jc.2004-0966]

104 **Miyata N**, Taniguchi K, Seki T, Ishimoto T, Sato-Watanabe M, Yasuda Y, Doi M, Kametani S, Tomishima Y, Ueki T, Sato M, Kameo K. HET0016, a potent and selective inhibitor of 20-HETE synthesizing enzyme. *Br J Pharmacol* 2001; **133**: 325-329 [PMID: 11375247 DOI: 10.1038/sj.bjp.0704101]

105 **Bishop-Bailey D**, Thomson S, Askari A, Faulkner A, Wheeler-Jones C. Lipid-metabolizing CYPs in the regulation and dysregulation of metabolism. *Annu Rev Nutr* 2014; **34**: 261-279 [PMID: 24819323 DOI: 10.1146/annurev-nutr-071813-105747]

106 **Lin YS**, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG, Thummel KE. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. *Mol Pharmacol* 2002; **62**: 162-172 [PMID: 12065767]

107 **Williams JA**, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, Hamman MA, Hall SD, Wrighton SA. Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos* 2002; **30**: 883-891 [PMID: 12124305]

108 **Onodera M**, Endo K, Kakuta Y, Kuroha M, Kimura T, Hiramoto K, Kanazawa Y, Negoro K, Shiga H, Kinouchi Y, Shimosegawa T. ATP-binding cassette subfamily B member 1 1236C/T polymorphism significantly affects the therapeutic outcome of tacrolimus in patients with refractory ulcerative colitis. *J Gastroenterol Hepatol* 2017; **32**: 1562-1569 [PMID: 28135009 DOI: 10.1111/jgh.13753]

109 **Onodera M**, Endo K, Naito T, Moroi R, Kuroha M, Kanazawa Y, Kimura T, Shiga H, Kakuta Y, Negoro K, Kinouchi Y, Shimosegawa T. Tacrolimus Dose Optimization Strategy for Refractory Ulcerative Colitis Based on the Cytochrome P450 3A5 Polymorphism Prediction Using Trough Concentration after 24 Hours. *Digestion* 2018; **97**: 90-96 [PMID: 29393157 DOI: 10.1159/000484227]

110 **Hirai F**, Takatsu N, Yano Y, Satou Y, Takahashi H, Ishikawa S, Tsurumi K, Hisabe T, Matsui T. Impact of CYP3A5 genetic polymorphisms on the pharmacokinetics and short-term remission in patients with ulcerative colitis treated with tacrolimus. *J Gastroenterol Hepatol* 2014; **29**: 60-66 [PMID: 24033383 DOI: 10.1111/jgh.12361]

111 **Naganuma M**, Fujii T, Watanabe M. The use of traditional and newer calcineurin inhibitors in inflammatory bowel disease. *J Gastroenterol* 2011; **46**: 129-137 [PMID: 21132334 DOI: 10.1007/s00535-010-0352-z]

112 **Cervelli M**, Russ G. Specialty practice series: intrapatient variability with tacrolimus. *Australian J Pharmacy* 2012; **93**: 83-86

113 **Kuehl P**, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 2001; **27**: 383-391 [PMID: 11279519 DOI: 10.1038/86882]

114 **Hesselink DA**, van Schaik RH, van der Heiden IP, van der Werf M, Gregoor PJ, Lindemans J, Weimar W,

van Gelder T. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. *Clin Pharmacol Ther* 2003; **74**: 245-254 [PMID: 12966368 DOI: 10.1016/S0009-9236(03)00168-1]

115 **Herrlinger KR**, Koc H, Winter S, Teml A, Stange EF, Fellermann K, Fritz P, Schwab M, Schaeffeler E. ABCB1 single-nucleotide polymorphisms determine tacrolimus response in patients with ulcerative colitis. *Clin Pharmacol Ther* 2011; **89**: 422-428 [PMID: 21289623 DOI: 10.1038/clpt.2010.348]

116 **Asada A**, Bamba S, Morita Y, Takahashi K, Imaeda H, Nishida A, Inatomi O, Sugimoto M, Sasaki M, Andoh A. The effect of CYP3A5 genetic polymorphisms on adverse events in patients with ulcerative colitis treated with tacrolimus. *Dig Liver Dis* 2017; **49**: 24-28 [PMID: 27717793 DOI: 10.1016/j.dld.2016.09.008]

117 **Lawrance IC**, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum Mol Genet* 2001; **10**: 445-456 [PMID: 11181568]

118 **Kim YH**, Bae YJ, Kim HS, Cha HJ, Yun JS, Shin JS, Seong WK, Lee YM, Han KM. Measurement of Human Cytochrome P450 Enzyme Induction Based on Mesalazine and Mosapride Citrate Treatments Using a Luminescent Assay. *Biomol Ther (Seoul)* 2015; **23**: 486-492 [PMID: 26336590 DOI: 10.4062/biomolther.2015.041]

119 **Kikuta Y**, Kato M, Yamashita Y, Miyauchi Y, Tanaka K, Kamada N, Kusunose M. Human leukotriene B4 omega-hydroxylase (CYP4F3) gene: molecular cloning and chromosomal localization. *DNA Cell Biol* 1998; **17**: 221-230 [PMID: 9539102 DOI: 10.1089/dna.1998.17.221]

120 **Corcos L**, Lucas D, Le Jossic-Corcos C, Dréano Y, Simon B, Plée-Gautier E, Amet Y, Salaün JP. Human cytochrome P450 4F3: structure, functions, and prospects. *Drug Metabol Drug Interact* 2012; **27**: 63-71 [PMID: 22706230 DOI: 10.1515/dmdi-2011-0037]

121 **Powell WS**, Rokach J. Biosynthesis, biological effects, and receptors of hydroxyeicosatetraenoic acids (HETEs) and oxoeicosatetraenoic acids (oxo-ETEs) derived from arachidonic acid. *Biochim Biophys Acta* 2015; **1851**: 340-355 [PMID: 25449650 DOI: 10.1016/j.bbalip.2014.10.008]

122 **Costea I**, Mack DR, Israel D, Morgan K, Krupoves A, Seidman E, Deslandres C, Lambrette P, Grimard G, Levy E, Amre DK. Genes involved in the metabolism of poly-unsaturated fatty-acids (PUFA) and risk for Crohn's disease in children & young adults. *PLoS One* 2010; **5**: e15672 [PMID: 21187935 DOI: 10.1371/journal.pone.0015672]

123 **Scaioli E**, Liverani E, Belluzzi A. The Imbalance between n-6/n-3 Polyunsaturated Fatty Acids and Inflammatory Bowel Disease: A Comprehensive Review and Future Therapeutic Perspectives. *Int J Mol Sci* 2017; **18** [PMID: 29206211 DOI: 10.3390/ijms18122619]

124 **Johnson AL**, Edson KZ, Totah RA, Rettie AE. Cytochrome P450 ω-Hydroxylases in Inflammation and Cancer. *Adv Pharmacol* 2015; **74**: 223-262 [PMID: 26233909 DOI: 10.1016/bs.apha.2015.05.002]

125 **Ananthakrishnan AN**, Khalili H, Song M, Higuchi LM, Lochhead P, Richter JM, Chan AT. Genetic Polymorphisms in Fatty Acid Metabolism Modify the Association Between Dietary n3: n6 Intake and Risk of Ulcerative Colitis: A Prospective Cohort Study. *Inflamm Bowel Dis* 2017; **23**: 1898-1904 [PMID: 28991856 DOI: 10.1097/MIB.0000000000001236]

126 **Yin J**, Liu H, Liu Z, Owzar K, Han Y, Su L, Wei Y, Hung RJ, Brhane Y, McLaughlin J, Brennan P, Bickeboeller H, Rosenberger A, Houlston RS, Caporaso N, Landi MT, Heinrich J, Risch A, Christiani DC, Amos CI, Wei Q. Pathway-analysis of published genome-wide association studies of lung cancer: A potential role for the CYP4F3 locus. *Mol Carcinog* 2017; **56**: 1663-1672 [PMID: 28150878 DOI: 10.1002/mc.22622]

127 **Jones G**, Prosser DE, Kaufmann M. Cytochrome P450-mediated metabolism of vitamin D. *J Lipid Res* 2014; **55**: 13-31 [PMID: 23564710 DOI: 10.1194/jlr.R031534]

128 **Jones G**, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. *Physiol Rev* 1998; **78**: 1193-1231 [PMID: 9790574 DOI: 10.1152/physrev.1998.78.4.1193]

129 **Prosser DE**, Jones G. Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci* 2004; **29**: 664-673 [PMID: 15544953 DOI: 10.1016/j.tibs.2004.10.005]

130 **Shinki T**, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K, Suda T. Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D3 in rat kidney but not in intestine. *J Biol Chem* 1992; **267**: 13757-13762 [PMID: 1618870]

131 **Shimada T**, Yamazaki Y, Takahashi M, Hasegawa H, Urakawa I, Oshima T, Ono K, Kakitani M, Tomizuka K, Fujita T, Fukumoto S, Yamashita T. Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism. *Am J Physiol Renal Physiol* 2005; **289**: F1088-F1095 [PMID: 15998839 DOI: 10.1152/ajprenal.00474.2004]

132 **Ramon I**, Kleynen P, Body JJ, Karmali R. Fibroblast growth factor 23 and its role in phosphate homeostasis. *Eur J Endocrinol* 2010; **162**: 1-10 [PMID: 19776202 DOI: 10.1530/EJE-09-0597]

133 **Rübsamen D**, Kunze MM, Buderus V, Brauß TF, Bajer MM, Brüne B, Schmid T. Inflammatory conditions induce IRES-dependent translation of cyp24a1. *PLoS One* 2014; **9**: e85314 [PMID: 24416388 DOI: 10.1371/journal.pone.0085314]

134 **Schlingmann KP**, Kaufmann M, Weber S, Irwin A, Goos C, John U, Misselwitz J, Klaus G, Kuwertz-Bröking E, Fehrenbach H, Wingen AM, Güran T, Hoenderop JG, Bindels RJ, Prosser DE, Jones G, Konrad M. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. *N Engl J Med* 2011; **365**: 410-421 [PMID: 21675912 DOI: 10.1056/NEJMoa1103864]

135 **Gong C**, Long Z, Yu Y, Zhu L, Tian J, Li S, Li J, Yu H, Chi Q, Piao D, Wang F, Zhao Y, Cui B. Dietary factors and polymorphisms in vitamin D metabolism genes: the risk and prognosis of colorectal cancer in northeast China. *Sci Rep* 2017; **7**: 8827 [PMID: 28821819 DOI: 10.1038/s41598-017-09356-1]

136 **Chen XQ**, Mao JY, Li WB, Li J, Yang H, Qian JM, Li JN. Association between *CYP24A1* polymorphisms and the risk of colonic polyps and colon cancer in a Chinese population. *World J Gastroenterol* 2017; **23**: 5179-5186 [PMID: 28811712 DOI: 10.3748/wjg.v23.i28.5179]

137 **Dong J**, Hu Z, Wu C, Guo H, Zhou B, Lv J, Lu D, Chen K, Shi Y, Chu M, Wang C, Zhang R, Dai J, Jiang Y, Cao S, Qin Z, Yu D, Ma H, Jin G, Gong J, Sun C, Zhao X, Yin Z, Yang L, Li Z, Deng Q, Wang J, Wu W, Zheng H, Zhou G, Chen H, Guan P, Peng Z, Chen Y, Shu Y, Xu L, Liu X, Liu L, Xu P, Han B, Bai C, Zhao Y, Zhang H, Yan Y, Amos CI, Chen F, Tan W, Jin L, Wu T, Lin D, Shen H. Association analyses identify multiple new lung cancer susceptibility loci and their interactions with smoking in the Chinese population. *Nat Genet* 2012; **44**: 895-899 [PMID: 22797725 DOI: 10.1038/ng.2351]

138 **Shiotani A**, Murao T, Fujita Y, Fujimura Y, Sakakibara T, Nishio K, Haruma K. Novel single nucleotide polymorphism markers for low dose aspirin-associated small bowel bleeding. *PLoS One* 2013; **8**: e84244 [PMID: 24367646 DOI: 10.1371/journal.pone.0084244]

139 **Kong J**, Xu F, Qu J, Wang Y, Gao M, Yu H, Qian B. Genetic polymorphisms in the vitamin D pathway in relation to lung cancer risk and survival. *Oncotarget* 2015; **6**: 2573-2582 [PMID: 25544771 DOI: 10.18632/oncotarget.2951]

140 **Zheng B**, Jeong S, Zhu Y, Chen L, Xia Q. miRNA and lncRNA as biomarkers in cholangiocarcinoma(CCA). *Oncotarget* 2017; **8**: 100819-100830 [PMID: 29246025 DOI: 10.18632/oncotarget.19044]

141 **Yu S**, Li X, Wang Y, Mao Z, Wang C, Ba Y, Li W. Transmission disequilibrium of rs4809957 in type 2 diabetes mellitus families and its association with vitamin D deficiency: A family-based case-control study. *J Diabetes Complications* 2018; **32**: 406-410 [PMID: 29428204 DOI: 10.1016/j.jdiacomp.2018.01.004]

142 **Pei FH**, Wang YJ, Gao SL, Liu BR, DU YJ, Liu W, Yu HY, Zhao LX, Chi BR. Vitamin D receptor gene polymorphism and ulcerative colitis susceptibility in Han Chinese. *J Dig Dis* 2011; **12**: 90-98 [PMID: 21401893 DOI: 10.1111/j.1751-2980.2011.00483.x]

143 **Hughes DJ**, McManus R, Neary P, O'morain C, O'sullivan M. Common variation in the vitamin D receptor gene and risk of inflammatory bowel disease in an Irish case-control study. *Eur J Gastroenterol Hepatol* 2011; **23**: 807-812 [PMID: 21818054 DOI: 10.1097/MEG.0b013e328349283e]

144 **Xue LN**, Xu KQ, Zhang W, Wang Q, Wu J, Wang XY. Associations between vitamin D receptor polymorphisms and susceptibility to ulcerative colitis and Crohn's disease: a meta-analysis. *Inflamm Bowel Dis* 2013; **19**: 54-60 [PMID: 22467262 DOI: 10.1002/ibd.22966]

145 **Cantorna MT**, Munsick C, Bemiss C, Mahon BD. 1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease. *J Nutr* 2000; **130**: 2648-2652 [PMID: 11053501 DOI: 10.1093/jn/130.11.2648]

146 **Di Rosa M**, Malaguarnera G, De Gregorio C, Palumbo M, Nunnari G, Malaguarnera L. Immuno-modulatory effects of vitamin D3 in human monocyte and macrophages. *Cell Immunol* 2012; **280**: 36-43 [PMID: 23261827 DOI: 10.1016/j.cellimm.2012.10.009]

147 **Nelson DR**. A second CYP26 P450 in humans and zebrafish: CYP26B1. *Arch Biochem Biophys* 1999; **371**: 345-347 [PMID: 10545224 DOI: 10.1006/abbi.1999.1438]

148 **Kang SG**, Wang C, Matsumoto S, Kim CH. High and low vitamin A therapies induce distinct FoxP3+ T-cell subsets and effectively control intestinal inflammation. *Gastroenterology* 2009; **137**: 1391-402.e1-6 [PMID: 19632226 DOI: 10.1053/j.gastro.2009.06.063]

149 **Takeuchi H**, Yokota A, Ohoka Y, Iwata M. Cyp26b1 regulates retinoic acid-dependent signals in T cells and its expression is inhibited by transforming growth factor-β. *PLoS One* 2011; **6**: e16089 [PMID: 21249211 DOI: 10.1371/journal.pone.0016089]

150 **Chenery A**, Burrows K, Antignano F, Underhill TM, Petkovich M, Zaph C. The retinoic acid-metabolizing enzyme Cyp26b1 regulates CD4 T cell differentiation and function. *PLoS One* 2013; **8**: e72308 [PMID: 23991089 DOI: 10.1371/journal.pone.0072308]

151 **Fransén K**, Franzén P, Magnuson A, Elmabsout AA, Nyhlin N, Wickbom A, Curman B, Törkvist L, D'Amato M, Bohr J, Tysk C, Sirsjö A, Halfvarson J. Polymorphism in the retinoic acid metabolizing enzyme CYP26B1 and the development of Crohn's Disease. *PLoS One* 2013; **8**: e72739 [PMID: 23977348 DOI: 10.1371/journal.pone.0072739]

152 **Holick MF**. Resurrection of vitamin D deficiency and rickets. *J Clin Invest* 2006; **116**: 2062-2072 [PMID: 16886050 DOI: 10.1172/JCI29449]

153 **Aranow C**. Vitamin D and the immune system. *J Investig Med* 2011; **59**: 881-886 [PMID: 21527855 DOI: 10.2310/JIM.0b013e31821b8755]

154 **Hahn HJ**, Kuttler B, Mathieu C, Bouillon R. 1,25-Dihydroxyvitamin D3 reduces MHC antigen expression on pancreatic beta-cells in vitro. *Transplant Proc* 1997; **29**: 2156-2157 [PMID: 9193569]

155 **Piemonti L**, Monti P, Sironi M, Fraticelli P, Leone BE, Dal Cin E, Allavena P, Di Carlo V. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol* 2000; **164**: 4443-4451 [PMID: 10779743]

156 **Lopez ER**, Zwermann O, Segni M, Meyer G, Reincke M, Seissler J, Herwig J, Usadel KH, Badenhoop K. A promoter polymorphism of the CYP27B1 gene is associated with Addison's disease, Hashimoto's thyroiditis, Graves' disease and type 1 diabetes mellitus in Germans. *Eur J Endocrinol* 2004; **151**: 193-197 [PMID: 15296474]

157 **Hewison M**, Burke F, Evans KN, Lammas DA, Sansom DM, Liu P, Modlin RL, Adams JS. Extra-renal 25-hydroxyvitamin D3-1alpha-hydroxylase in human health and disease. *J Steroid Biochem Mol Biol* 2007; **103**: 316-321 [PMID: 17368179 DOI: 10.1016/j.jsbmb.2006.12.078]

158 **Adams JS**, Rafison B, Witzel S, Reyes RE, Shieh A, Chun R, Zavala K, Hewison M, Liu PT. Regulation of the extrarenal CYP27B1-hydroxylase. *J Steroid Biochem Mol Biol* 2014; **144** Pt A: 22-27 [PMID: 24388948 DOI: 10.1016/j.jsbmb.2013.12.009]

159 **Du J**, Wei X, Ge X, Chen Y, Li YC. Microbiota-Dependent Induction of Colonic Cyp27b1 Is Associated With Colonic Inflammation: Implications of Locally Produced 1,25-Dihydroxyvitamin D3 in Inflammatory Regulation in the Colon. *Endocrinology* 2017; **158**: 4064-4075 [PMID: 28938443 DOI: 10.1210/en.2017-00578]

160 **Alzahrani AS**, Zou M, Baitei EY, Alshaikh OM, Al-Rijjal RA, Meyer BF, Shi Y. A novel G102E mutation of CYP27B1 in a large family with vitamin D-dependent rickets type 1. *J Clin Endocrinol Metab* 2010; **95**: 4176-4183 [PMID: 20534770 DOI: 10.1210/jc.2009-2278]

161 **Fichna M**, Zurawek M, Januszkiewicz-Lewandowska D, Gryczyñska M, Fichna P, Sowiñski J, Nowak J. Association of the CYP27B1 C(-1260)A polymorphism with autoimmune Addison's disease. *Exp Clin Endocrinol Diabetes* 2010; **118**: 544-549 [PMID: 19998245 DOI: 10.1055/s-0029-1241206]

162 **Ramagopalan SV**, Dyment DA, Cader MZ, Morrison KM, Disanto G, Morahan JM, Berlanga-Taylor AJ, Handel A, De Luca GC, Sadovnick AD, Lepage P, Montpetit A, Ebers GC. Rare variants in the CYP27B1 gene are associated with multiple sclerosis. *Ann Neurol* 2011; **70**: 881-886 [PMID: 22190362 DOI: 10.1002/ana.22678]

163 **Ross JP**, Bernales CQ, Lee JD, Sadovnick AD, Traboulsee AL, Vilariño-Güell C. Analysis of CYP27B1 in multiple sclerosis. *J Neuroimmunol* 2014; **266**: 64-66 [PMID: 24308945 DOI: 10.1016/j.jneuroim.2013.11.006]

164 **Demir K**, Kattan WE, Zou M, Durmaz E, BinEssa H, Nalbantoğlu Ö, Al-Rijjal RA, Meyer B, Özkan B, Shi Y. Novel CYP27B1 Gene Mutations in Patients with Vitamin D-Dependent Rickets Type 1A. *PLoS One* 2015; **10**: e0131376 [PMID: 26132292 DOI: 10.1371/journal.pone.0131376]

165 **Gould NJ**, Davidson KL, Nwokolo CU, Arasaradnam RP. A systematic review of the role of DNA methylation on inflammatory genes in ulcerative colitis. *Epigenomics* 2016; **8**: 667-684 [PMID: 27096966

DOI: 10.2217/epi-2016-0006]
166 **Barnicle A**, Seoighe C, Greally JM, Golden A, Egan LJ. Inflammation-associated DNA methylation patterns in epithelium of ulcerative colitis. *Epigenetics* 2017; **12**: 591-606 [PMID: 28557546 DOI: 10.1080/15592294.2017.1334023]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2863-2877

DOI: 10.3748/wjg.v25.i23.2863

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Role of epigenetics in transformation of inflammation into colorectal cancer

Zhen-Hua Yang, Yan-Qi Dang, Guang Ji

ORCID number: Zhen-Hua Yang (0000-0001-9844-6411); Yan-Qi Dang (0000-0001-8651-6937); Guang Ji (0000-0003-0842-3676).

Author contributions: All authors contributed equally to this paper with regard to conception and design of the research, literature review and analysis, manuscript critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: The authors declare no conflict of interests for this article.

Manuscript source: Unsolicited manuscript

Received: March 2, 2019
Peer-review started: March 2, 2019
First decision: April 16, 2019
Revised: April 24, 2019
Accepted: May 8, 2019
Article in press: May 8, 2019
Published online: June 21, 2019

P-Reviewer: Das U, Sterpetti AV,

Zhen-Hua Yang, Yan-Qi Dang, Guang Ji, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Zhen-Hua Yang, Digestive Endoscopy Department, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

Corresponding author: Guang Ji, MD, PhD, Chief Physician, Director, Doctor, Full Professor, Occupational Physician, Professor, Research Scientist, Teacher, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, China. jiliver@vip.sina.com
Telephone: +86-21-64385700
Fax: +86-21-64398310

Abstract

Molecular mechanisms associated with inflammation-promoted tumorigenesis have become an important topic in cancer research. Various abnormal epigenetic changes, including DNA methylation, histone modification, chromatin remodeling, and noncoding RNA regulation, occur during the transformation of chronic inflammation into colorectal cancer (CRC). These changes not only accelerate transformation but also lead to cancer progression and metastasis by activating carcinogenic signaling pathways. The NF-κB and STAT3 signaling pathways play a particularly important role in the transformation of inflammation into CRC, and both are critical to cellular signal transduction and constantly activated in cancer by various abnormal changes including epigenetics. The NF-κB and STAT3 signals contribute to the microenvironment for tumorigenesis through secretion of a large number of pro-inflammatory cytokines and their crosstalk in the nucleus makes it even more difficult to treat CRC. Compared with gene mutation that is irreversible, epigenetic inheritance is reversible or can be altered by the intervention. Therefore, understanding the role of epigenetic inheritance in the inflammation-cancer transformation may elucidate the pathogenesis of CRC and promote the development of innovative drugs targeting transformation to prevent and treat this malignancy. This review summarizes the literature on the roles of epigenetic mechanisms in the occurrence and development of inflammation-induced CRC. Exploring the role of epigenetics in the transformation of inflammation into CRC may help stimulate futures studies on the role of molecular therapy in CRC.

Key words: Colorectal cancer; Inflammation; DNA methylation; Histone modification; LncRNA; MicroRNAs; Epigenetics

Vetvicka V, Zheng YW
S-Editor: Yan JP
L-Editor: Wang TQ
E-Editor: Ma YJ

Core tip: Chronic inflammation can promote the occurrence and progression of colorectal cancer (CRC), drawing more attention to the role of pro-inflammatory cytokines and immune cells in this cancer. By regulating the expression of various inflammatory signaling pathways and pro-inflammatory cytokines, epigenetic inheritance not only participates in the transformation of inflammation into CRC, but also facilitates invasion, metastasis, and drug resistance of CRC. Compared with gene mutation that is irreversible, epigenetic inheritance is reversible or can be altered by the intervention. Therefore, the tumor microenvironment can be regulated by modulating epigenetic modifications, which may be novel alternatives to prevent and treat CRC.

Citation: Yang ZH, Dang YQ, Ji G. Role of epigenetics in transformation of inflammation into colorectal cancer. *World J Gastroenterol* 2019; 25(23): 2863-2877
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2863.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2863

INTRODUTION

In recent years, accumulating evidence indicates that chronic inflammation leads to the occurrence and development of many tumors[1,2]. The relationship between inflammation and cancer has long been investigated. Two thousand years ago, the Greek physician Galen described the similarities between cancer and inflammation and believed that cancer might evolve from inflammatory lesions[3]. In 1863, Virchow identified inflammatory cell infiltration in tumor tissues and explained it as a reaction to the origination of cancer at sites of chronic inflammation, proposing the hypothesis of the inflammation-cancer transformation[4]. Colorectal cancer (CRC) is the third most common malignant tumor and the fourth leading cause of cancer-related mortality worldwide[5]. The pathogenesis of CRC is complex. Previously, most scholars believed that CRC was a genopathy and a highly heterogeneous tumor resulting from an accumulation of genetic abnormalities, the failure of cancer defense mechanisms, and the activation of carcinogenic pathways[6]. However, increasing evidence suggests that CRC is a typical inflammation-dependent cancer. The risk of developing CRC increases in patients with inflammatory bowel disease (IBD), such as ulcerative colitis or Crohn's disease, which is more likely to be caused by chronic inflammation of the intestinal mucosa than by any definitive genetic predisposition[7-9]. In addition, chronic inflammation plays an important role in the occurrence and development of sporadic CRC, and the expression of interleukin-1 (IL-1), IL-6, IL-17A, and IL-23 is increased in most sporadic CRC cases[10,11].

Various proinflammatory signaling pathways participate in the transformation of inflammation into CRC, including the NF-κB, IL-6/STAT3, cyclooxygenase-2 (COX-2)/PGE_2, and IL-23/Th17 pathways, which induce the production of inflammatory mediators, upregulate the expression of antiapoptotic genes, stimulate cell proliferation and angiogenesis, and thereby contribute to tumorigenesis[12]. The NF-κB signaling pathway includes both classical and non-canonical pathways. The classical pathway is activated by pro-inflammatory cytokines, pathogen-associated or damage-associated molecular patterns. The non-canonical pathway is activated by a small subset of cytokines including lymphotoxin, receptor activator of NF-κB ligand, CD40 ligand, and B cell activating factor of the tumor necrosis factor (TNF) family[13]. Activation of NF-κB not only affects DNA damage and carcinogenic mutations, but also causes tumorigenesis by promoting the production of reactive oxygen species (ROS) and reactive nitrogen. It can also cause chromosomal instability, aneuploidy, and epigenetic changes, leading to tumorigenesis and development[14,15]. NF-κB and STAT3 are nuclear transcription factors required for the regulation of tumor proliferation, survival, angiogenesis, and invasion; their target genes encode the critical cancer-promoting inflammatory mediators[13-19]. NF-κB and STAT3 signaling contributes to the tumorigenic microenvironment by mediating the secretion of various proinflammatory cytokines, and the crosstalk of these pathways in the nucleus makes CRC even more difficult to treat[18,20,21].

To date, abundant evidence has indicated that epigenetic changes play an important role in the transformation of inflammation into CRC as well as in the occurrence, development, invasion, metastasis, and drug resistance of this cancer.

These epigenetic changes include DNA methylation, histone modification, and noncoding RNA (ncRNA) alterations. Molecular mechanisms associated with inflammation-promoted tumorigenesis are currently an important branch of cancer research; therefore, understanding the role of epigenetic inheritance in the occurrence and development of CRC may elucidate the pathogenesis of this cancer and promote the development of innovative drugs targeting transformation for CRC treatment.

DNA METHYLATION

DNA methylation is an important epigenetic modification related to gene expression and mediated by DNA methyltransferases (DNMTs), and an imbalance in genomic methylation leads to tumors. Approximately half of human gene promoters are rich in C-G sequences, also called CpG loci because of the phosphodiester bond linking the C and G nucleotides. If they are present in DNA sequences, CpG islands are likely located in genetic regulatory elements[22,23] and are usually defined as regions with a length greater than 200 base pairs and a G + C content greater than 50%[24,25]. DNA methylation starts at one end of the islands and continues to gene promoters and initiation sites, altering the three-dimensional configuration of the DNA and inhibiting its interaction with transcription factors, ultimately silencing gene expression. In contrast, hypomethylation promotes gene expression[23,26]. In CRC, the commonly observed types of methylation include hypermethylation of antioncogene DNA and hypomethylation of oncogene DNA. More importantly, DNA methylation can be stably inherited by progeny cells through histone marks at methylation sites, leading to hereditary effects without changes in DNA sequences[27].

IBD has been demonstrated to increase the risk of CRC incidence. A meta-analysis of population-based cohort studies indicated that the incidence of CRC within 10, 20, and > 20 years was 1%, 2%, and 5%, respectively, in patients with IBD[28,29]. In addition, DNA methylation promotes the tumorigenesis and progression of colitis-associated CRC (CAC). The expression of DNMT1 is appreciably higher in CAC samples than in tumor tissues of patients with sporadic CRC, indicating an increased level of DNA methylation in CAC tissues[30]. However, the frequency of chromosomal instability and microsatellite instability in CAC is generally the same as that in sporadic CRC. In approximately 46% of patients with microsatellite instability-high CAC, the hMLH1 gene is hypermethylated[31]. The cell cycle inhibitor gene $p16^{INK4a}$, which has been reported to be negatively associated with the occurrence of sporadic CRC, is often methylated in tumor samples from CAC patients[32,33]. In addition, the $p14^{ARF}$ gene can indirectly regulate the expression of the p53 protein, and methylation of $p14^{ARF}$ is a relatively common early event in ulcerative colitis-associated colorectal carcinogenesis[34]. Furthermore, the methylation levels of the ITGA4, TFPI2, and VIM gene promoters are increased in inflamed colon tissues, which may imply a high risk of CAC development[35] (Figure 1A). Recent genome-wide studies have provided important insights into the characteristics of DNA methylation in tumors[36]. Using monoalkyl methylation profiles in a colitis-induced mouse colon cancer model, Abu-Remaileh *et al*[37] identified a novel epigenetic modification characterized by hypermethylation of the DNA methylation valley (DMV), which leads to the silencing of DMV-related genes, thus facilitating inflammation-induced cell transformation.

Studies have shown that the methylation of specific genes is associated with inflammatory conditions, dysplasia, and malignant transformation, indicating that epigenetic modifications are involved in inflammation-induced carcinogenesis[38-40]. Many proinflammatory cytokines secreted as a result of NF-κB and STAT3 signaling pathway activation are activated and promote the transformation of inflammation into CRC[20]. For example, IL-6 silences the expression of suppressor of cytokine signaling 3 (SOCS 3) by inducing high expression levels of DNMT1. SOCS3 is an important negative regulator of cytokine-induced STAT3 signaling, and its silencing ultimately contributes to the occurrence of CRC[41]. TNF molecules depend on the NF-κB signaling pathway to silence the gene encoding the proapoptotic protein kinase cδ-binding protein through gene promoter methylation, which facilitates the growth of cancer cells[42]. IL-6 has been demonstrated to increase the methylation of the promoter regions of genes related to tumor inhibition, cell adhesion, and apoptosis resistance, and this increase could be prevented by treatment with the DNMT 1 inhibitor 5-azadeoxycytidine[30] (Figure 1B). IL-6 produced during intestinal inflammation can modulate the expression of CYP2E1 and CYP1B1 (cytochrome P450 enzymes) *via* transcriptional and epigenetic mechanisms, altering the metabolic capability of epithelial cells. Indeed, one study suggested that IL-6 reduces the expression of miR27b, which targets CYP1B1, through a DNA methylation mechanism, thereby increasing dietary carcinogen activation and DNA injury, which leads to the

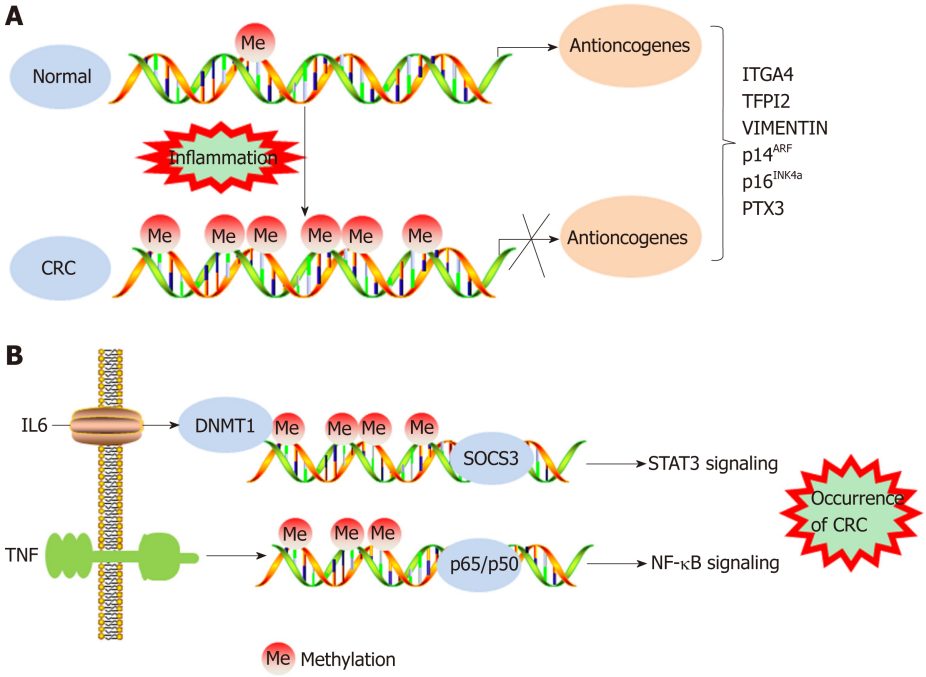


Figure 1 DNA methylation regulates the transformation of inflammation into colorectal cancer. A: DNA hypermethylation levels inhibit expression of antioncogenes, resulting in the occurrence of colorectal cancer (CRC); B: Inflammatory cytokines regulate STAT3/NF-κB signaling to promote the occurrence of CRC by DNA methylation. CRC: Colorectal cancer; IL-6: Interleukin-6; DNMT1: DNA methyltransferase 1; SOCS3: Suppressor of cytokine signaling 3.

occurrence of CRC[43]. As an important component of natural humoral immunity, PTX3 activates and regulates the complement cascade by interacting with C1q and factor H and plays a role in the regulation of inflammation. PTX3 has been considered an exogenous antioncogene, and PTX3 deficiency increases sensitivity to epithelial carcinogenesis[44,45]. An analysis of epigenomic data revealed high methylation levels in the PTX3 gene promoter in CRC[46,47]. Prostaglandin, a signaling molecule with important pro- and anti-inflammatory effects, is synthesized from arachidonic acid through the prostaglandin endoperoxide synthase (PTGS; also called cyclooxygenase or COX) pathway. PTGS2 (also called COX-2), one of the key enzymes in the pathway, is overexpressed in CRC, leading to oversecretion of the downstream metabolite prostaglandin E2 (PGE-2)[48]. Deregulation of the COX-2/PGE_2 signaling pathway is associated with many tumors, including CRC, and the expression levels of COX-2 and PGE_2 are closely related not only to metastasis and poor prognosis in patients with CRC but also to chemotherapeutic resistance in tumors[49-52]. Indeed, a study showed that high methylation rates of select gene promoters stimulate the production of PGE_2, block the production of other bioactive prostaglandins, and ultimately promote the development of CRC[53]. Moreover, the results of this study suggest that the antitumor effects of nonsteroidal anti-inflammatory drugs (NSAIDs) may be related to the ability of these drugs to inhibit COX-2. FXR regulates bile acid metabolism and inhibits the production of the secondary bile acid cholic acid; therefore, FXR performs anticancer functions. In CRC, the expression of FXR is negatively associated with the degree of tumor malignancy and with poor clinical outcomes[54,55]. The APC gene is typically mutationally inactivated in the pathogenesis of CRC[56]. Loss of function of APC silences FXR expression through CpG methylation in mouse colonic mucosa and human colon cells, decreasing the expression of downstream bile acid-binding proteins and heterodimers and increasing the expression of related genes (COX-2 and c-MYC) in inflammation and CRC[57]. Recent studies demonstrated that vitamin D (VD) deficiency is associated with the occurrence of CRC. VD, an anti-inflammatory agent, regulates adipocytes and their functions *via* the VD receptor (VDR), resulting in decreased expression of proinflammatory cytokines[58-61]. Using blood and visceral adipose tissues collected from CRC patients and healthy controls, Castellano-Castillo *et al*[62] explored the relationship among the levels of serum 25-hydroxyvitamin D [25(OH)D], expression of the VDR gene in adipose tissue, levels of proinflammatory markers, expression of the epigenetic factor DNMT3A, and methylation of the VDR promoter. These results suggest that adipose tissue may be a critical factor in the occurrence of CRC and that low expression levels of 25(OH)D and high expression levels of VDR may partially mediate this relationship by modulating DNA methylation and promoting inflammation[62]. In addition, inflammatory mediators

such as ROS and reactive nitrogen species may lead to genomic instability, which contributes to carcinogenesis *via* the mutation of protooncogenes and tumor suppressor genes[63]. Vitamin C (VC) and vitamin E (VE) are antioxidants that can scavenge free radicals[64,65]. One study suggests that VE antagonizes high glucose-induced oxidative stress, exhibiting beneficial effects on gene promoter methylation and gene expression in the CRC cell line Caco-2[66]. In addition, the results of an *in vitro* experiment indicated that VC could enhance antitumor drug-induced DNA hydroxymethylation and reactivate epigenetically silenced expression of the tumor suppressor CDKN1A in CRC cells[67]. Therefore, supplementation with related vitamins may be an alternative approach to treat CRC. Moreover, black raspberry (BRB) anthocyanins, which can modulate changes in inflammation and SFRP2 gene methylation, have been reported as agents for CRC prevention[68]. In summary, DNA methylation facilitates the transformation of inflammation into CRC in both the local environment of intestinal inflammation and systemic inflammation.

HISTONE MODIFICATION

Chromatin is a macromolecular complex composed of DNA, RNA, and proteins. Histones, which regulate DNA strand compaction and gene expression, are the main protein component of chromatin[23]. The core histones are composed of four major families-H2A, H2B, H3, and H4[69]. The nucleosome is a chromatin unit consisting of 150 to 200 base pairs of DNA wrapped closely around a cylindrical histone core. Posttranslational covalent modification of the histone tail constitutes an epigenetic mechanism that regulates chromatin structure and gene expression in human cancers. Histone tail modifications include phosphorylation, methylation, acetylation, ubiquitination, glycosylation, deamination, and ribosylation[70,71]. Various modifications alter the three-dimensional structure of nucleosomes and affect the transcriptional control of related genes by inducing either an "inactive" tight heterochromatin or an "active" open euchromatin conformation. Insight into histone modification is not as deep as that into DNA methylation; the only well-studied histone modifications are the acetylation/deacetylation and methylation/demethylation of lysine and arginine residues in the histone tail[72,73]. These bivalent histone modifications are mediated by polycomb group proteins (transcriptional repressors), which contribute to silencing a specific set of anti-oncogenes in human cancers. Polycomb repressive complexes (PRCs) include PRC1 and PRC2, which silence genes alone or in cooperation[74,75].

Histone modification abnormalities arise during the transformation of inflammation into CRC. Profiles for active enhancers using H3K27ac histone modification identified several cancer markers, such as the LYZ, S100P, and NPSR1 proteins, which were elevated in CAC[76]. In a mouse model, deletion of the Giα2 protein led to spontaneous colitis and right multifocal CRC, which is similar to human CRC with defective mismatch repair. Moreover, MLH1 and PMS2 expression are reduced in the colonic epithelium of Giα2$^{-/-}$ mice after the onset of hypoxic colitis. Notably, MLH1 is epigenetically silenced by reduced histone acetylation. These data connect chronic hypoxic inflammation, histone modulation, and CRC development[77]. The Wnt/β-catenin signaling pathway exerts either pro- or anti-inflammatory functions by activating or inhibiting NF-κB signals, respectively, playing an important role in the occurrence and development of CRC[78]. DACT3, the negative regulator of Wnt/beta-catenin signaling, is transcriptionally suppressed in CRC in a manner related to bivalent histone modification. However, DACT3 expression can be restored after combined administration of drugs targeting histone methylation and deacetylation, resulting in strong inhibition of Wnt/beta-catenin signal transduction and massive CRC cell apoptosis. Therefore, DACT3 may be an important factor in the treatment of CRC through epigenetic mechanisms[79]. EZH2, a catalytic subunit of PRC2, is essential for maintaining the integrity and homeostasis of the epithelial cell barrier in inflammatory states. EZH2 expression is downregulated in IBD patients, and EZH2 inactivation in the intestinal epithelium increases the sensitivity of mice to dextran sodium sulfate (DSS)- and 2,4,6-trinitrobenzenesulfonic acid-induced experimental colitis. One study indicated that EZH2 deficiency could stimulate the expression of TRAF2/5 and enhance the NF-κB signaling induced by TNF-α, which led to an uncontrolled inflammatory reaction and ultimately contributed to tumorigenesis[80]. Researchers identified a new epigenetic mechanism underlying the preventive effects of aspirin in CAC. Aspirin reduced the activity of histone deacetylases and fully restored H3K27ac. Moreover, aspirin depressed azoxymethane/DSS-induced H3K27ac accumulation in the promoters of the inducible nitric oxide synthase, TNF-α, and IL-6 genes and suppressed the production of proin-

flammatory cytokines, playing a role in the prevention of cancer[81] (Figure 2). Although few studies have investigated the role of histone modification in the transformation of inflammation into CRC, notably, histone modification may interact with DNA methylation to induce epigenetic silencing and promote tumorigenesis.

MICRORNAS

Most of the human genome is transcribed into RNAs, which are classified as RNAs with coding potential or RNAs without. The latter RNAs are also called ncRNAs. NcRNAs were historically considered "transcriptional waste", but accumulating evidence indicates that ncRNAs strongly impact many molecular mechanisms[82]. MicroRNAs are single-stranded ncRNAs with a length of 20 nucleotides that function primarily to negatively regulate gene expression by binding to target RNAs and inducing the degradation or inhibiting the translation of those RNAs[83].

The NF-κB and STAT3 signaling pathways play an important role in the transformation of inflammation into cancer[13], and numerous microRNAs promote transformation by participating in these signaling pathways. Slattery *et al*[84] analyzed the expression profiles of genes and associated microRNAs in the NF-κB signaling pathway between CRC and normal mucosa, providing new insight into therapeutic targets for CRC. TNF-α has been shown to increase the expression of miR-105, which targets RAP2C, activate NF-κB signal transduction by IKK, and ultimately contribute to CRC progression[85]. Another study demonstrated that TNF-α leads to high expression of miR-19a, which can also activate NF-κB signaling to facilitate the occurrence of colitis and CAC[86]. STAT3 not only is a downstream target of IL-6 but also interacts with miR-21, miR-181b-1, PTEN, and CYLD, which implicates the epigenetic switch that links inflammation to cancer[87]. In addition, by activating miR-21, NR2F2 inhibits Smad7 expression and promotes TGF-β-dependent epithelial-mesenchymal transition (EMT) in CRC[88]. In primary CRC samples and cell lines, increased STAT3 expression levels are accompanied by elevated miR-572 and decreased MOAP-1 levels, and miR-572 has been found to reduce the expression of the proapoptotic protein MOAP-1 and to contribute to CRC progression[89]. Öner *et al*[90] demonstrated that combined inactivation of TP53 and miR-34a promotes CRC metastasis by elevating the levels of IL-6R and PAI1, implying that modifying these processes might be alternative approaches to treat CRC. Immune cells play a dual role in the occurrence and development of CRC, and natural killer (NK) cells promote tumor cell apoptosis by secreting high levels of cytokines, such as IFN-γ and TNF-α[91]. The level of miR-24 was reported to be increased in NK cells from CRC patients, a characteristic that decreased the levels of cytokines, including IFN-γ and TNF-α, by suppressing Paxillin expression and inhibiting the cytotoxic effect of NK cells on CRC cells[92].

MicroRNAs not only promote the transformation of inflammation into CRC but also lead to chemotherapeutic resistance. For example, in CRC patients treated with oxaliplatin, miR-34a expression decreased significantly but Smad4 and TGF-β expression increased. In addition, the expression levels of Smad4 and miR-34a were negatively correlated in CRC patients. Further investigation demonstrated that miR-34a targeted Smad4 through the TGF-β/Smad4 pathway and ultimately inhibited cellular autophagy[93]. Ren *et al* found overexpression of miR-196b-5p in recurrent CRC tissues, which was associated with a poor prognosis. Further investigation showed that miR-196b-5p activated STAT3 signaling and promoted the chemical resistance of CRC cells to 5-fluorouracil (5-FU) by targeting the negative regulators SOCS1 and SOCS3 in the STAT3 signaling pathway[94]. Clarification of the role of microRNAs in CRC resistance mechanisms can improve the efficacy of chemotherapy by targeting the corresponding microRNAs. Indeed, microRNAs overexpressed during the transformation of inflammation into CRC are often associated with disease progression. In addition, certain microRNAs are silenced or downregulated during the occurrence and development of CRC; these microRNAs generally inhibit the transformation of inflammation into cancer. For instance, miR-148a, miR-6869-5p, and miR-139-5p inhibit transformation by suppressing the NF-κB signaling pathway[95-97]; miR-1299, miR-149, and miR-214 inhibit tumor formation by suppressing the STAT3 signaling pathway[98-100]; and miR-329 inhibits CRC occurrence by targeting TGF-β1[101]. MiR15A and miR16-1, which might act as tumor suppressors, were found to be downregulated in CRC[102]. Animal experiments showed that deficiency of miR15A and miR16-1 led to an accumulation of immunosuppressive IgA^+ B cells in intestinal cancer tissues, thereby inhibiting the proliferation and functions of $CD8^+$ T cells with antitumor immunity and ultimately promoting tumor progression[103].

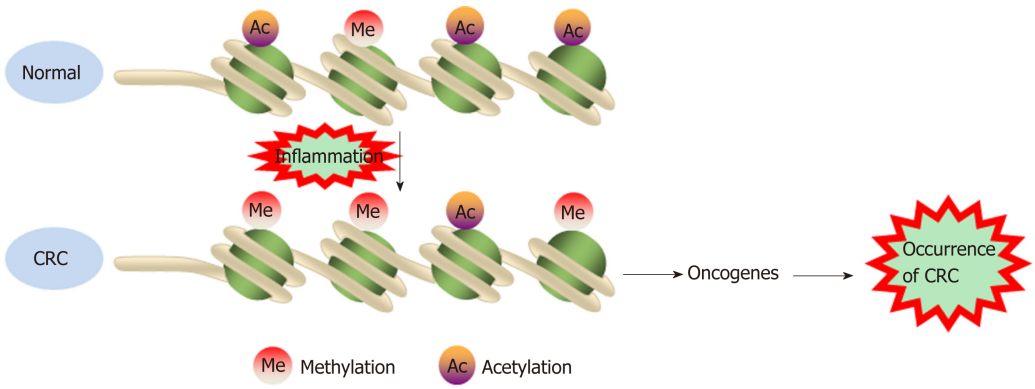


Figure 2 Histone methylation and acetylation modifications increase oncogene expression to promote cancer occurrence. CRC: Colorectal cancer.

Exosomal microRNAs

Exosomes are small vesicles released by cells into the extracellular environment. These vesicles transmit information to adjacent or distant cells by transferring RNAs and proteins, thus affecting signaling pathways in various physiological and pathological conditions[104,105]. Recently, microRNAs enriched in exosomes have been found to interact with immune cells and proinflammatory cytokines and to participate in the progression and metastasis of CRC. For example, exosome-mediated miR-200b was indicated to promote the proliferation of CRC cells upon TGF-β1 exposure[106]. In addition, it was reported that the level of miR-10b was significantly higher in exosomes derived from CRC cells than in those derived from normal colorectal epithelial cells and that CRC-derived exosomes could promote CRC progression[107]. Tumor-associated macrophages (TAMs), a biomarker of solid tumors, are usually associated with a poor prognosis. A recent study has shown that CRC cells carrying mutant p53 genes selectively shed miR-1246-enriched exosomes. These exosomes can be captured by neighboring TAMs, which then secrete numerous proinflammatory cytokines, such as IL-10, TGF-β, and MMPs, to promote the immune suppression, invasion, and metastasis of CRC cells[108] (Figure 3, Table 1).

LNCRNAS

LncRNAs refer to noncoding transcripts of more than 200 nucleotides[109]. LncRNAs are important participants in cancer biology and generally cause the abnormal expression of gene products, leading to the progression of various human tumors[110,111]. In addition, lncRNAs are involved in the transformation of chronic inflammation into CRC. For example, a study indicated that the interaction between lncRNA PRINS and miR-491-5p regulated the proapoptotic factor PMAIP1 and enhanced the antiapoptotic effect of TFF3 against the proapoptotic effects of IFN-γ and TNF-α in CRC cells[112]. LncRNA FEZF1-AS1 expression was higher in CRC tissue than in normal tissue and was associated with a poor prognosis in CRC. FEZF1-AS1 can bind to and increase the stability of the pyruvate kinase 2 (PKM2) protein, which can increase PKM2 levels in the cytoplasm and nucleus and promote pyruvate kinase activity and lactic acid production. Upregulation of nuclear PKM2 induced by FEZF1-AS1 was found to further activate STAT3 signal transduction and accelerate the transformation of inflammation to cancer[113]. In addition, researchers found that lncRNA AB073614 induced EMT in CRC cells by regulating the JAK/STAT3 pathway[114]. Recent studies have shown that lncRNAs not only participate in the transformation of inflammation to tumors but also induce the resistance of CRC to chemotherapy by regulating inflammatory signaling pathways. Abnormal expression of HOTAIR is positively correlated with progression, survival, and poor prognosis in different types of cancers, such as breast cancer, gastric cancer, and CRC[115-117]. Li *et al*[118] revealed that HOTAIR silenced the expression of the miR-218 gene by recruiting EZH2 for binding to the miR-218 promoter. Silencing of miR-218 resulted in chemotherapeutic resistance of CRC to 5-FU by promoting VOPP1 expression and eventually activating the NF-kB/TS signaling pathway. HOTAIR can thus be used as a novel prognostic indicator and therapeutic target for CRC. Inhibiting HOTAIR may be a future approach to improve the sensitivity of 5-FU chemotherapy (Figure 4, Table 2).

Table 1 MicroRNAs regulate inflammation and occurrence of colorectal cancer

MicroRNAs	Target genes	Functions in CRC
MiR-105, miR-19a,	NF-κB	Promoted CRC progression
MiR-21, miR-181b-1, miR-572	STAT3	Promoted CRC progression
MiR-21, miR-200b, miR-1246	TGF-β	Promoted proliferation
MiR-34a	IL-6R/PAI1	Promoted CRC progression
MiR-24	IFN-γ/TNF-α	Inhibited the cytotoxic effect of NK cells
MiR-34a	TGF-β/Smad4	Inhibited autophagy
MiR-196b-5p	STAT3	Promoted chemical resistance
MiR-148a, miR-6869-5p, miR-139-5p	NF-κB	Inhibited CRC occurrence
MiR-1299, miR-149, miR-214	STAT3	Inhibited tumor formation
MiR-329	TGF-β1	Inhibited CRC occurrence

CRC: Colorectal cancer; NK: Natural killer.

ANTI-INFLAMMATORY AGENTS AND CRC

Due to the inflammatory basis of CRC, anti-inflammatory agents may be candidates for treating or preventing the disease. NSAIDs are non-selective inhibitors of COX-2[119]. COX-2 is highly expressed in many tumor types, including CRC[120]. NSAIDs play a striking role in the prevention of CRC. A preliminary study on patients with familial adenomatous polyposis indicated that after 1 year of treatment with the NSAID sulindac, patients tended to exhibit a decrease in polyps[121]. A large-scale observational study in 1991 reported that the use of NSAIDs reduced the risk of fatal CRC[122]. Retrospective studies have demonstrated that NSAID treatment is associated with a decreased risk of recurrence of colorectal polyps and tumors. It has been reported that patients who use low-dose aspirin for more than 5 years show a decrease in overall risk of CRC by 40%-50%, and NSAIDs have a positive effect on advanced CRC[123,124]. NSAID therapy can also inhibit the tumor-promoting pathway by inhibiting Wnt signaling[125]. However, a meta-analysis of NSAID treatment to prevent the transformation of IBD into CRC shows that there is a lack of high-quality evidence that anti-inflammatory drugs can be used to prevent CRC in patients with IBD[126]. Most scholars believe that the anti-tumor mechanism of NSAIDs is reflected in two aspects. On the one hand, the cytokines released during inflammation play an important role in reprogramming adult stem cells into malignant cells, and NSAIDs can prevent this process[127]. On the other hand, the mechanism is related to activation of the Wnt pathway by prostaglandins[128]. However, the side effects of NSAIDs must be taken into consideration. NSAID treatment can increase the risk of gastrointestinal bleeding, even at low doses[129]. Caution should be exercised to prevent bleeding when using anti-inflammatory drugs in patients with fragile blood vessels[130]. In addition to NSAIDs, monoclonal antibodies to cytokines such as IL-6 and TNF inhibitors have been investigated in a large number of anti-tumor studies, but most of them are still in the experimental stage[131,132]. The treatment hazard of using anti-cytokines to treat tumors is the increase in the risk of infection[133,134].

CONCLUSION

In conclusion, chronic inflammation can promote the occurrence and progression of CRC, a finding that is attracting increased attention to the role of proinflammatory cytokines and immune cells in cancer. By regulating the expression of various inflammatory signaling pathways and proinflammatory cytokines, epigenetic inheritance not only participates in the transformation of inflammation into CRC but also facilitates CRC invasion, metastasis, and drug resistance. However, epigenetic inheritance accelerates the transformation of chronic inflammation into tumors through various modifications influenced by the inflammatory environment. An in-depth understanding of this process will allow us to clarify the pathogenesis of CRC, and some epigenetic modifications can be used as markers for CRC diagnosis. Unlike gene mutations, which are irreversible, epigenetic inheritance is reversible or can be altered by interventions. For example, DNA demethylation promotes tumor suppressor gene expression to re-establish tumor prevention and reduce expression of pro-inflammatory cytokines by regulation of histone modifications or ncRNAs,

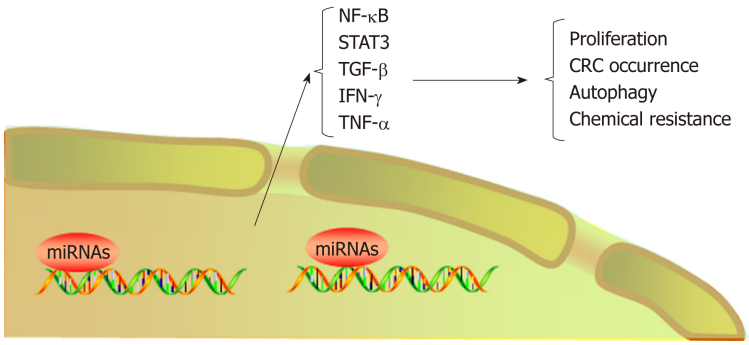


Figure 3 MicroRNAs regulate colorectal cancer progression by regulating inflammatory cytokines. CRC: Colorectal cancer; TNF-α: Tumor necrosis factor α; TGF-β: Transforming growth factor β.

ultimately reducing inflammation infiltration of tumor microenvironment. In the future, this new anti-tumor drug may be used in combination with immunotherapy, chemotherapy, and targeted cancer therapy for the treatment of CRC. Exploring the role of epigenetics in the transformation of inflammation into CRC may help stimulate futures studies on the role of molecular therapy in CRC.

Table 2 LncRNAs regulate the inflammation-cancer transformation

LncRNA	Target genes	Functions in CRC
PRINS	miR-491-5p/PMAIP1/TFF3	Inhibited apoptosis
FEZF1-AS1	PKM2/STAT3	Accelerated CRC occurrence
AB073614	JAK/STAT3	Induced EMT
HOTAIR	MiR-218/EZH2/NF-kB	Chemotherapeutic resistance

CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition.

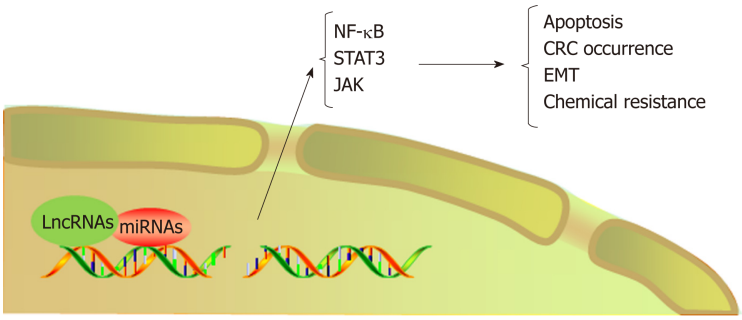


Figure 4 LncRNAs regulate the occurrence and chemotherapeutic resistance of colorectal cancer by mediating microRNAs/inflammatory signaling pathways. CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition; JAK: Janus kinase.

REFERENCES

1 **Hanahan D**, Weinberg RA. Hallmarks of cancer: The next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
2 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]
3 **Reedy J**. Galen on cancer and related diseases. *Clio Med* 1975; **10**: 227-238 [PMID: 50913]
4 **Balkwill F**, Mantovani A. Inflammation and cancer: Back to Virchow? *Lancet* 2001; **357**: 539-545 [PMID: 11229684 DOI: 10.1016/s0140-6736(00)04046-0]
5 **Brenner H**, Kloor M, Pox CP. Colorectal cancer. *Lancet* 2014; **383**: 1490-1502 [PMID: 24225001 DOI: 10.1016/S0140-6736(13)61649-9]
6 **Vaiopoulos AG**, Kostakis ID, Koutsilieris M, Papavassiliou AG. Colorectal cancer stem cells. *Stem Cells* 2012; **30**: 363-371 [PMID: 22232074 DOI: 10.1002/stem.1031]
7 **Ullman TA**, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology* 2011; **140**: 1807-1816 [PMID: 21530747 DOI: 10.1053/j.gastro.2011.01.057]
8 **Pedersen N**, Duricova D, Elkjaer M, Gamborg M, Munkholm P, Jess T. Risk of extra-intestinal cancer in inflammatory bowel disease: Meta-analysis of population-based cohort studies. *Am J Gastroenterol* 2010; **105**: 1480-1487 [PMID: 20332773 DOI: 10.1038/ajg.2009.760]
9 **Bernstein CN**, Blanchard JF, Rawsthorne P, Yu N. The prevalence of extraintestinal diseases in inflammatory bowel disease: A population-based study. *Am J Gastroenterol* 2001; **96**: 1116-1122 [PMID: 11316157 DOI: 10.1111/j.1572-0241.2001.03756.x]
10 **Tosolini M**, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pagès F, Galon J. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res* 2011; **71**: 1263-1271 [PMID: 21303976 DOI: 10.1158/0008-5472.CAN-10-2907]
11 **Schetter AJ**, Nguyen GH, Bowman ED, Mathé EA, Yuen ST, Hawkes JE, Croce CM, Leung SY, Harris CC. Association of inflammation-related and microRNA gene expression with cancer-specific mortality of colon adenocarcinoma. *Clin Cancer Res* 2009; **15**: 5878-5887 [PMID: 19737943 DOI: 10.1158/1078-0432.CCR-09-0627]
12 **Luo C**, Zhang H. The Role of Proinflammatory Pathways in the Pathogenesis of Colitis-Associated Colorectal Cancer. *Mediators Inflamm* 2017; **2017**: 5126048 [PMID: 28852270 DOI: 10.1155/2017/5126048]
13 **Taniguchi K**, Karin M. NF-κB, inflammation, immunity and cancer: Coming of age. *Nat Rev Immunol* 2018; **18**: 309-324 [PMID: 29379212 DOI: 10.1038/nri.2017.142]
14 **Karin M**, Greten FR. NF-kappaB: Linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**: 749-759 [PMID: 16175180 DOI: 10.1038/nri1703]
15 **Baud V**, Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov* 2009; **8**: 33-40 [PMID: 19116625 DOI: 10.1038/nrd2781]
16 **Darnell JE**. Transcription factors as targets for cancer therapy. *Nat Rev Cancer* 2002; **2**: 740-749 [PMID: 12360277 DOI: 10.1038/nrc906]
17 **Yu H**, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer* 2004; **4**: 97-105 [PMID: 14964307 DOI: 10.1038/nrc1275]
18 **Yu H**, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: Role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* 2007; **7**: 41-51 [PMID: 17186030 DOI: 10.1038/nri1995]

19 **Bassères DS**, Baldwin AS. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* 2006; **25**: 6817-6830 [PMID: 17072330 DOI: 10.1038/sj.onc.1209942]

20 **West NR**, McCuaig S, Franchini F, Powrie F. Emerging cytokine networks in colorectal cancer. *Nat Rev Immunol* 2015; **15**: 615-629 [PMID: 26358393 DOI: 10.1038/nri3896]

21 **Grivennikov SI**, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev* 2010; **21**: 11-19 [PMID: 20018552 DOI: 10.1016/j.cytogfr.2009.11.005]

22 **Goel A**, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology* 2012; **143**: 1442-1460.e1 [PMID: 23000599 DOI: 10.1053/j.gastro.2012.09.032]

23 **Okugawa Y**, Grady WM, Goel A. Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. *Gastroenterology* 2015; **149**: 1204-1225.e12 [PMID: 26216839 DOI: 10.1053/j.gastro.2015.07.011]

24 **Gardiner-Garden M**, Frommer M. CpG islands in vertebrate genomes. *J Mol Biol* 1987; **196**: 261-282 [PMID: 3656447 DOI: 10.1016/0022-2836(87)90689-9]

25 **Lao VV**, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 686-700 [PMID: 22009203 DOI: 10.1038/nrgastro.2011.173]

26 **Graff JR**, Herman JG, Myöhänen S, Baylin SB, Vertino PM. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J Biol Chem* 1997; **272**: 22322-22329 [PMID: 9268383 DOI: 10.1074/jbc.272.35.22322]

27 **Ooi SL**, Henikoff S. Germline histone dynamics and epigenetics. *Curr Opin Cell Biol* 2007; **19**: 257-265 [PMID: 17467256 DOI: 10.1016/j.ceb.2007.04.015]

28 **Beaugerie L**, Itzkowitz SH. Cancers Complicating Inflammatory Bowel Disease. *N Engl J Med* 2015; **373**: 195 [PMID: 26154801 DOI: 10.1056/NEJMc1505689]

29 **Lutgens MW**, van Oijen MG, van der Heijden GJ, Vleggaar FP, Siersema PD, Oldenburg B. Declining risk of colorectal cancer in inflammatory bowel disease: An updated meta-analysis of population-based cohort studies. *Inflamm Bowel Dis* 2013; **19**: 789-799 [PMID: 23448792 DOI: 10.1097/MIB.0b013e31828029c0]

30 **Foran E**, Garrity-Park MM, Mureau C, Newell J, Smyrk TC, Limburg PJ, Egan LJ. Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. *Mol Cancer Res* 2010; **8**: 471-481 [PMID: 20354000 DOI: 10.1158/1541-7786.MCR-09-0496]

31 **Fleisher AS**, Esteller M, Harpaz N, Leytin A, Rashid A, Xu Y, Liang J, Stine OC, Yin J, Zou TT, Abraham JM, Kong D, Wilson KT, James SP, Herman JG, Meltzer SJ. Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1. *Cancer Res* 2000; **60**: 4864-4868 [PMID: 10987299 DOI: 10.1016/S0016-5085(00)84957-4]

32 **Wang FY**, Arisawa T, Tahara T, Takahama K, Watanabe M, Hirata I, Nakano H. Aberrant DNA methylation in ulcerative colitis without neoplasia. *Hepatogastroenterology* 2008; **55**: 62-65 [PMID: 18507080]

33 **Hsieh CJ**, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. *Cancer Res* 1998; **58**: 3942-3945 [PMID: 9731506]

34 **Sato F**, Harpaz N, Shibata D, Xu Y, Yin J, Mori Y, Zou TT, Wang S, Desai K, Leytin A, Selaru FM, Abraham JM, Meltzer SJ. Hypermethylation of the p14(ARF) gene in ulcerative colitis-associated colorectal carcinogenesis. *Cancer Res* 2002; **62**: 1148-1151 [PMID: 11861396 DOI: 10.1002/cncr.10308]

35 **Gerecke C**, Scholtka B, Löwenstein Y, Fait I, Gottschalk U, Rogoll D, Melcher R, Kleuser B. Hypermethylation of ITGA4, TFPI2 and VIMENTIN promoters is increased in inflamed colon tissue: Putative risk markers for colitis-associated cancer. *J Cancer Res Clin Oncol* 2015; **141**: 2097-2107 [PMID: 25902909 DOI: 10.1007/s00432-015-1972-8]

36 **Berman BP**, Weisenberger DJ, Aman JF, Hinoue T, Ramjan Z, Liu Y, Noushmehr H, Lange CP, van Dijk CM, Tollenaar RA, Van Den Berg D, Laird PW. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet* 2011; **44**: 40-46 [PMID: 22120008 DOI: 10.1038/ng.969]

37 **Abu-Remaileh M**, Bender S, Raddatz G, Ansari I, Cohen D, Gutekunst J, Musch T, Linhart H, Breiling A, Pikarsky E, Bergman Y, Lyko F. Chronic inflammation induces a novel epigenetic program that is conserved in intestinal adenomas and in colorectal cancer. *Cancer Res* 2015; **75**: 2120-2130 [PMID: 25808873 DOI: 10.1158/0008-5472.CAN-14-3295]

38 **Hahn MA**, Hahn T, Lee DH, Esworthy RS, Kim BW, Riggs AD, Chu FF, Pfeifer GP. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. *Cancer Res* 2008; **68**: 10280-10289 [PMID: 19074896 DOI: 10.1158/0008-5472.CAN-08-1957]

39 **Wehbe H**, Henson R, Meng F, Mize-Berge J, Patel T. Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. *Cancer Res* 2006; **66**: 10517-10524 [PMID: 17079474 DOI: 10.1158/0008-5472.Can-06-2130]

40 **Issa JP**, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001; **61**: 3573-3577 [PMID: 11325821 DOI: 10.1046/j.1523-5394.2001.009003155.x]

41 **Li Y**, Deuring J, Peppelenbosch MP, Kuipers EJ, de Haar C, van der Woude CJ. IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer. *Carcinogenesis* 2012; **33**: 1889-1896 [PMID: 22739025 DOI: 10.1093/carcin/bgs214]

42 **Lee JH**, Kang MJ, Han HY, Lee MG, Jeong SI, Ryu BK, Ha TK, Her NG, Han J, Park SJ, Lee KY, Kim HJ, Chi SG. Epigenetic alteration of PRKCDBP in colorectal cancers and its implication in tumor cell resistance to TNFα-induced apoptosis. *Clin Cancer Res* 2011; **17**: 7551-7562 [PMID: 21980136 DOI: 10.1158/1078-0432.CCR-11-1026]

43 **Patel SA**, Bhambra U, Charalambous MP, David RM, Edwards RJ, Lightfoot T, Boobis AR, Gooderham NJ. Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3. *Br J Cancer* 2014; **111**: 2287-2296 [PMID: 25333344 DOI: 10.1038/bjc.2014.540]

44 **Bottazzi B**, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: Pentraxins as a paradigm. *Annu Rev Immunol* 2010; **28**: 157-183 [PMID: 19968561 DOI: 10.1146/annurev-immunol-030409-101305]

45 **Deban L**, Jarva H, Lehtinen MJ, Bottazzi B, Bastone A, Doni A, Jokiranta TS, Mantovani A, Meri S.

Binding of the long pentraxin PTX3 to factor H: Interacting domains and function in the regulation of complement activation. *J Immunol* 2008; **181**: 8433-8440 [PMID: 19050261 DOI: 10.4049/jimmunol.181.12.8433]

46 **Bonavita E**, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, Greco C, Feruglio F, Molgora M, Laface I, Tartari S, Doni A, Pasqualini F, Barbati E, Basso G, Galdiero MR, Nebuloni M, Roncalli M, Colombo P, Laghi L, Lambris JD, Jaillon S, Garlanda C, Mantovani A. PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. *Cell* 2015; **160**: 700-714 [PMID: 25679762 DOI: 10.1016/j.cell.2015.01.004]

47 **Rubino M**, Kunderfranco P, Basso G, Greco CM, Pasqualini F, Serio S, Roncalli M, Laghi L, Mantovani A, Papait R, Garlanda C. Epigenetic regulation of the extrinsic oncosuppressor PTX3 gene in inflammation and cancer. *Oncoimmunology* 2017; **6**: e1333215 [PMID: 28811977 DOI: 10.1080/2162402X.2017.1333215]

48 **Wang D**, Dubois RN. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 2010; **29**: 781-788 [PMID: 19946329 DOI: 10.1038/onc.2009.421]

49 **Greenhough A**, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, Kaidi A. The COX-2/PGE2 pathway: Key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009; **30**: 377-386 [PMID: 19136477 DOI: 10.1093/carcin/bgp014]

50 **Cathcart MC**, Lysaght J, Pidgeon GP. Eicosanoid signalling pathways in the development and progression of colorectal cancer: Novel approaches for prevention/intervention. *Cancer Metastasis Rev* 2011; **30**: 363-385 [PMID: 22134655 DOI: 10.1007/s10555-011-9324-x]

51 **Ma X**, Aoki T, Tsuruyama T, Narumiya S. Definition of Prostaglandin E2-EP2 Signals in the Colon Tumor Microenvironment That Amplify Inflammation and Tumor Growth. *Cancer Res* 2015; **75**: 2822-2832 [PMID: 26018088 DOI: 10.1158/0008-5472.CAN-15-0125]

52 **Kunzmann AT**, Murray LJ, Cardwell CR, McShane CM, McMenamin UC, Cantwell MM. PTGS2 (Cyclooxygenase-2) expression and survival among colorectal cancer patients: A systematic review. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 1490-1497 [PMID: 23810915 DOI: 10.1158/1055-9965.EPI-13-0263]

53 **Cebola I**, Custodio J, Muñoz M, Díez-Villanueva A, Paré L, Prieto P, Aussó S, Coll-Mulet L, Boscá L, Moreno V, Peinado MA. Epigenetics override pro-inflammatory PTGS transcriptomic signature towards selective hyperactivation of PGE2 in colorectal cancer. *Clin Epigenetics* 2015; **7**: 74 [PMID: 26207152 DOI: 10.1186/s13148-015-0110-4]

54 **Lax S**, Schauer G, Prein K, Kapitan M, Silbert D, Berghold A, Berger A, Trauner M. Expression of the nuclear bile acid receptor/farnesoid X receptor is reduced in human colon carcinoma compared to nonneoplastic mucosa independent from site and may be associated with adverse prognosis. *Int J Cancer* 2012; **130**: 2232-2239 [PMID: 21780109 DOI: 10.1002/ijc.26293]

55 **Modica S**, Gadaleta RM, Moschetta A. Deciphering the nuclear bile acid receptor FXR paradigm. *Nucl Recept Signal* 2010; **8**: e005 [PMID: 21383957 DOI: 10.1621/nrs.08005]

56 **Markowitz SD**, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009; **361**: 2449-2460 [PMID: 20018966 DOI: 10.1056/NEJMra0804588]

57 **Selmin OI**, Fang C, Lyon AM, Doetschman TC, Thompson PA, Martinez JD, Smith JW, Lance PM, Romagnolo DF. Inactivation of Adenomatous Polyposis Coli Reduces Bile Acid/Farnesoid X Receptor Expression through Fxr gene CpG Methylation in Mouse Colon Tumors and Human Colon Cancer Cells. *J Nutr* 2016; **146**: 236-242 [PMID: 26609171 DOI: 10.3945/jn.115.216580]

58 **Saccone D**, Asani F, Bornman L. Regulation of the vitamin D receptor gene by environment, genetics and epigenetics. *Gene* 2015; **561**: 171-180 [PMID: 25682935 DOI: 10.1016/j.gene.2015.02.024]

59 **Song M**, Konijeti GG, Yuan C, Ananthakrishnan AN, Ogino S, Fuchs CS, Giovannucci EL, Ng K, Chan AT. Plasma 25-Hydroxyvitamin D, Vitamin D Binding Protein, and Risk of Colorectal Cancer in the Nurses' Health Study. *Cancer Prev Res (Phila)* 2016; **9**: 664-672 [PMID: 27246684 DOI: 10.1158/1940-6207.CAPR-16-0053]

60 **Ding C**, Gao D, Wilding J, Trayhurn P, Bing C. Vitamin D signalling in adipose tissue. *Br J Nutr* 2012; **108**: 1915-1923 [PMID: 23046765 DOI: 10.1017/S0007114512003285]

61 **Lira FS**, Rosa JC, Cunha CA, Ribeiro EB, do Nascimento CO, Oyama LM, Mota JF. Supplementing alpha-tocopherol (vitamin E) and vitamin D3 in high fat diet decrease IL-6 production in murine epididymal adipose tissue and 3T3-L1 adipocytes following LPS stimulation. *Lipids Health Dis* 2011; **10**: 37 [PMID: 21352586 DOI: 10.1186/1476-511X-10-37]

62 **Castellano-Castillo D**, Morcillo S, Clemente-Postigo M, Crujeiras AB, Fernandez-García JC, Torres E, Tinahones FJ, Macias-Gonzalez M. Adipose tissue inflammation and VDR expression and methylation in colorectal cancer. *Clin Epigenetics* 2018; **10**: 60 [PMID: 29719581 DOI: 10.1186/s13148-018-0493-0]

63 **Schetter AJ**, Heegaard NH, Harris CC. Inflammation and cancer: Interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* 2010; **31**: 37-49 [PMID: 19955394 DOI: 10.1093/carcin/bgp272]

64 **Serbinova E**, Kagan V, Han D, Packer L. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. *Free Radic Biol Med* 1991; **10**: 263-275 [PMID: 1649783 DOI: 10.1016/0891-5849(91)90033-Y]

65 **Du J**, Cullen JJ, Buettner GR. Ascorbic acid: Chemistry, biology and the treatment of cancer. *Biochim Biophys Acta* 2012; **1826**: 443-457 [PMID: 22728050 DOI: 10.1016/j.bbcan.2012.06.003]

66 **Zappe K**, Pointner A, Switzeny OJ, Magnet U, Tomeva E, Heller J, Mare G, Wagner KH, Knasmueller S, Haslberger AG. Counteraction of Oxidative Stress by Vitamin E Affects Epigenetic Regulation by Increasing Global Methylation and Gene Expression of MLH1 and DNMT1 Dose Dependently in Caco-2 Cells. *Oxid Med Cell Longev* 2018; **2018**: 3734250 [PMID: 29854080 DOI: 10.1155/2018/3734250]

67 **Gerecke C**, Schumacher F, Edlich A, Wetzel A, Yealland G, Neubert LK, Scholtka B, Homann T, Kleuser B. Vitamin C promotes decitabine or azacytidine induced DNA hydroxymethylation and subsequent reactivation of the epigenetically silenced tumour suppressor CDKN1A in colon cancer cells. *Oncotarget* 2018; **9**: 32822-32840 [PMID: 30214687 DOI: 10.18632/oncotarget.25999]

68 **Chen L**, Jiang B, Zhong C, Guo J, Zhang L, Mu T, Zhang Q, Bi X. Chemoprevention of colorectal cancer by black raspberry anthocyanins involved the modulation of gut microbiota and SFRP2 demethylation. *Carcinogenesis* 2018; **39**: 471-481 [PMID: 29361151 DOI: 10.1093/carcin/bgy009]

69 **Andrews AJ**, Luger K. Nucleosome structure(s) and stability: Variations on a theme. *Annu Rev Biophys* 2011; **40**: 99-117 [PMID: 21332355 DOI: 10.1146/annurev-biophys-042910-155329]

70 **Kouzarides T**. Chromatin modifications and their function. *Cell* 2007; **128**: 693-705 [PMID: 17320507 DOI: 10.1016/j.cell.2007.02.005]

71 **Jenuwein T**, Allis CD. Translating the histone code. *Science* 2001; **293**: 1074-1080 [PMID: 11498575 DOI: 10.1126/science.1063127]
72 **Jones PA**, Baylin SB. The epigenomics of cancer. *Cell* 2007; **128**: 683-692 [PMID: 17320506 DOI: 10.1016/j.cell.2007.01.029]
73 **Chen HP**, Zhao YT, Zhao TC. Histone deacetylases and mechanisms of regulation of gene expression. *Crit Rev Oncog* 2015; **20**: 35-47 [PMID: 25746103 DOI: 10.1615/CritRevOncog.2015012997]
74 **Hahn MA**, Li AX, Wu X, Yang R, Drew DA, Rosenberg DW, Pfeifer GP. Loss of the polycomb mark from bivalent promoters leads to activation of cancer-promoting genes in colorectal tumors. *Cancer Res* 2014; **74**: 3617-3629 [PMID: 24786786 DOI: 10.1158/0008-5472.CAN-13-3147]
75 **Sparmann A**, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 2006; **6**: 846-856 [PMID: 17060944 DOI: 10.1038/nrc1991]
76 **Sarvestani SK**, Signs SA, Lefebvre V, Mack S, Ni Y, Morton A, Chan ER, Li X, Fox P, Ting A, Kalady MF, Cruise M, Ashburn J, Stiene J, Lai W, Liska D, Xiang S, Huang EH. Cancer-predicting transcriptomic and epigenetic signatures revealed for ulcerative colitis in patient-derived epithelial organoids. *Oncotarget* 2018; **9**: 28717-28730 [PMID: 29983891 DOI: 10.18632/oncotarget.25617]
77 **Edwards RA**, Witherspoon M, Wang K, Afrasiabi K, Pham T, Birnbaumer L, Lipkin SM. Epigenetic repression of DNA mismatch repair by inflammation and hypoxia in inflammatory bowel disease-associated colorectal cancer. *Cancer Res* 2009; **69**: 6423-6429 [PMID: 19638594 DOI: 10.1158/0008-5472.CAN-09-1285]
78 **Ma B**, Hottiger MO. Crosstalk between Wnt/β-Catenin and NF-κB Signaling Pathway during Inflammation. *Front Immunol* 2016; **7**: 378 [PMID: 27713747 DOI: 10.3389/fimmu.2016.00378]
79 **Jiang X**, Tan J, Li J, Kivimäe S, Yang X, Zhuang L, Lee PL, Chan MT, Stanton LW, Liu ET, Cheyette BN, Yu Q. DACT3 is an epigenetic regulator of Wnt/beta-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. *Cancer Cell* 2008; **13**: 529-541 [PMID: 18538736 DOI: 10.1016/j.ccr.2008.04.019]
80 **Liu Y**, Peng J, Sun T, Li N, Zhang L, Ren J, Yuan H, Kan S, Pan Q, Li X, Ding Y, Jiang M, Cong X, Tan M, Ma Y, Fu D, Cai S, Xiao Y, Wang X, Qin J. Epithelial EZH2 serves as an epigenetic determinant in experimental colitis by inhibiting TNFα-mediated inflammation and apoptosis. *Proc Natl Acad Sci U S A* 2017; **114**: E3796-E3805 [PMID: 28439030 DOI: 10.1073/pnas.1700909114]
81 **Guo Y**, Liu Y, Zhang C, Su ZY, Li W, Huang MT, Kong AN. The epigenetic effects of aspirin: The modification of histone H3 lysine 27 acetylation in the prevention of colon carcinogenesis in azoxymethane- and dextran sulfate sodium-treated CF-1 mice. *Carcinogenesis* 2016; **37**: 616-624 [PMID: 27207670 DOI: 10.1093/carcin/bgw042]
82 **ENCODE Project Consortium**. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012; **489**: 57-74 [PMID: 22955616 DOI: 10.1038/nature11247]
83 **Castel SE**, Martienssen RA. RNA interference in the nucleus: Roles for small RNAs in transcription, epigenetics and beyond. *Nat Rev Genet* 2013; **14**: 100-112 [PMID: 23329111 DOI: 10.1038/nrg3355]
84 **Slattery ML**, Mullany LE, Sakoda L, Samowitz WS, Wolff RK, Stevens JR, Herrick JS. The NF-κB signalling pathway in colorectal cancer: Associations between dysregulated gene and miRNA expression. *J Cancer Res Clin Oncol* 2018; **144**: 269-283 [PMID: 29188362 DOI: 10.1007/s00432-017-2548-6]
85 **Shen Z**, Zhou R, Liu C, Wang Y, Zhan W, Shao Z, Liu J, Zhang F, Xu L, Zhou X, Qi L, Bo F, Ding Y, Zhao L. MicroRNA-105 is involved in TNF-α-related tumor microenvironment enhanced colorectal cancer progression. *Cell Death Dis* 2017; **8**: 3213 [PMID: 29238068 DOI: 10.1038/s41419-017-0048-x]
86 **Wang T**, Xu X, Xu Q, Ren J, Shen S, Fan C, Hou Y. miR-19a promotes colitis-associated colorectal cancer by regulating tumor necrosis factor alpha-induced protein 3-NF-κB feedback loops. *Oncogene* 2017; **36**: 3240-3251 [PMID: 27991929 DOI: 10.1038/onc.2016.468]
87 **Iliopoulos D**, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell* 2010; **39**: 493-506 [PMID: 20797623 DOI: 10.1016/j.molcel.2010.07.023]
88 **Wang H**, Nie L, Wu L, Liu Q, Guo X. NR2F2 inhibits Smad7 expression and promotes TGF-β-dependent epithelial-mesenchymal transition of CRC via transactivation of miR-21. *Biochem Biophys Res Commun* 2017; **485**: 181-188 [PMID: 28192117 DOI: 10.1016/j.bbrc.2017.02.049]
89 **Wang N**, He X, Zhou R, Jia G, Qiao Q. STAT3 induces colorectal carcinoma progression through a novel miR-572-MOAP-1 pathway. *Onco Targets Ther* 2018; **11**: 3475-3484 [PMID: 29942139 DOI: 10.2147/OTT.S158764]
90 **Öner MG**, Rokavec M, Kaller M, Bouznad N, Horst D, Kirchner T, Hermeking H. Combined Inactivation of TP53 and MIR34A Promotes Colorectal Cancer Development and Progression in Mice Via Increasing Levels of IL6R and PAI1. *Gastroenterology* 2018; **155**: 1868-1882 [PMID: 30099074 DOI: 10.1053/j.gastro.2018.08.011]
91 **Wang R**, Jaw JJ, Stutzman NC, Zou Z, Sun PD. Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1. *J Leukoc Biol* 2012; **91**: 299-309 [PMID: 22045868 DOI: 10.1189/jlb.0611308]
92 **Zhang LL**, Zhang LF, Shi YB. miR-24 inhibited the killing effect of natural killer cells to colorectal cancer cells by downregulating Paxillin. *Biomed Pharmacother* 2018; **101**: 257-263 [PMID: 29494963 DOI: 10.1016/j.biopha.2018.02.024]
93 **Sun C**, Wang FJ, Zhang HG, Xu XZ, Jia RC, Yao L, Qiao PF. miR-34a mediates oxaliplatin resistance of colorectal cancer cells by inhibiting macroautophagy via transforming growth factor-β/Smad4 pathway. *World J Gastroenterol* 2017; **23**: 1816-1827 [PMID: 28348487 DOI: 10.3748/wjg.v23.i10.1816]
94 **Ren D**, Lin B, Zhang X, Peng Y, Ye Z, Ma Y, Liang Y, Cao L, Li X, Li R, Sun L, Liu Q, Wu J, Zhou K, Zeng J. Maintenance of cancer stemness by miR-196b-5p contributes to chemoresistance of colorectal cancer cells via activating STAT3 signaling pathway. *Oncotarget* 2017; **8**: 49807-49823 [PMID: 28591704 DOI: 10.18632/oncotarget.17971]
95 **Zhu Y**, Gu L, Li Y, Lin X, Shen H, Cui K, Chen L, Zhou F, Zhao Q, Zhang J, Zhong B, Prochownik E, Li Y. miR-148a inhibits colitis and colitis-associated tumorigenesis in mice. *Cell Death Differ* 2017; **24**: 2199-2209 [PMID: 28960206 DOI: 10.1038/cdd.2017.151]
96 **Yan S**, Liu G, Jin C, Wang Z, Duan Q, Xu J, Xu D. MicroRNA-6869-5p acts as a tumor suppressor via targeting TLR4/NF-κB signaling pathway in colorectal cancer. *J Cell Physiol* 2018; **233**: 6660-6668 [PMID: 29206292 DOI: 10.1002/jcp.26316]
97 **Zou F**, Mao R, Yang L, Lin S, Lei K, Zheng Y, Ding Y, Zhang P, Cai G, Liang X, Liu J. Targeted deletion of miR-139-5p activates MAPK, NF-κB and STAT3 signaling and promotes intestinal inflammation and colorectal cancer. *FEBS J* 2016; **283**: 1438-1452 [PMID: 26859226 DOI: 10.1111/febs.13678]

98 **Wang AL**, Li Y, Zhao Q, Fan LQ. Formononetin inhibits colon carcinoma cell growth and invasion by microRNA149mediated EphB3 downregulation and inhibition of PI3K/AKT and STAT3 signaling pathways. *Mol Med Rep* 2018; **17**: 7721-7729 [PMID: 29620230 DOI: 10.3892/mmr.2018.8857]

99 **Wang Y**, Lu Z, Wang N, Zhang M, Zeng X, Zhao W. MicroRNA-1299 is a negative regulator of STAT3 in colon cancer. *Oncol Rep* 2017; **37**: 3227-3234 [PMID: 28498395 DOI: 10.3892/or.2017.5605]

100 **Chandrasekaran KS**, Sathyanarayanan A, Karunagaran D. miR-214 activates TP53 but suppresses the expression of RELA, CTNNB1, and STAT3 in human cervical and colorectal cancer cells. *Cell Biochem Funct* 2017; **35**: 464-471 [PMID: 29023799 DOI: 10.1002/cbf.3304]

101 **Li B**, Huang M, Liu M, Wen S, Sun F. MicroRNA329 serves a tumor suppressive role in colorectal cancer by directly targeting transforming growth factor beta1. *Mol Med Rep* 2017; **16**: 3825-3832 [PMID: 29067459 DOI: 10.3892/mmr.2017.7077]

102 **Huang E**, Liu R, Chu Y. miRNA-15a/16: As tumor suppressors and more. *Future Oncol* 2015; **11**: 2351-2363 [PMID: 26260813 DOI: 10.2217/fon.15.101]

103 **Liu R**, Lu Z, Gu J, Liu J, Huang E, Liu X, Wang L, Yang J, Deng Y, Qian J, Luo F, Wang Z, Zhang H, Jiang X, Zhang D, Qian J, Liu G, Zhu H, Qian Y, Liu Z, Chu Y. MicroRNAs 15A and 16-1 Activate Signaling Pathways That Mediate Chemotaxis of Immune Regulatory B cells to Colorectal Tumors. *Gastroenterology* 2018; **154**: 637-651.e7 [PMID: 29031499 DOI: 10.1053/j.gastro.2017.09.045]

104 **Kosaka N**, Yoshioka Y, Fujita Y, Ochiya T. Versatile roles of extracellular vesicles in cancer. *J Clin Invest* 2016; **126**: 1163-1172 [PMID: 26974161 DOI: 10.1172/JCI81130]

105 **Roma-Rodrigues C**, Fernandes AR, Baptista PV. Exosome in tumour microenvironment: Overview of the crosstalk between normal and cancer cells. *Biomed Res Int* 2014; **2014**: 179486 [PMID: 24963475 DOI: 10.1155/2014/179486]

106 **Zhang Z**, Xing T, Chen Y, Xiao J. Exosome-mediated miR-200b promotes colorectal cancer proliferation upon TGF-β1 exposure. *Biomed Pharmacother* 2018; **106**: 1135-1143 [PMID: 30119180 DOI: 10.1016/j.biopha.2018.07.042]

107 **Dai G**, Yao X, Zhang Y, Gu J, Geng Y, Xue F, Zhang J. Colorectal cancer cell-derived exosomes containing miR-10b regulate fibroblast cells via the PI3K/Akt pathway. *Bull Cancer* 2018; **105**: 336-349 [PMID: 29496262 DOI: 10.1016/j.bulcan.2017.12.009]

108 **Cooks T**, Pateras IS, Jenkins LM, Patel KM, Robles AI, Morris J, Forshew T, Appella E, Gorgoulis VG, Harris CC. Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246. *Nat Commun* 2018; **9**: 771 [PMID: 29472616 DOI: 10.1038/s41467-018-03224-w]

109 **Mattick JS**, Rinn JL. Discovery and annotation of long noncoding RNAs. *Nat Struct Mol Biol* 2015; **22**: 5-7 [PMID: 25565026 DOI: 10.1038/nsmb.2942]

110 **Ma Y**, Yang Y, Wang F, Moyer MP, Wei Q, Zhang P, Yang Z, Liu W, Zhang H, Chen N, Wang H, Wang H, Qin H. Long non-coding RNA CCAL regulates colorectal cancer progression by activating Wnt/β-catenin signalling pathway via suppression of activator protein 2α. *Gut* 2016; **65**: 1494-1504 [PMID: 25994219 DOI: 10.1136/gutjnl-2014-308392]

111 **Fu M**, Zou C, Pan L, Liang W, Qian H, Xu W, Jiang P, Zhang X. Long noncoding RNAs in digestive system cancers: Functional roles, molecular mechanisms, and clinical implications (Review). *Oncol Rep* 2016; **36**: 1207-1218 [PMID: 27431376 DOI: 10.3892/or.2016.4929]

112 **Buda A**, Jepson MA, Pignatelli M. Regulatory function of trefoil peptides (TFF) on intestinal cell junctional complexes. *Cell Commun Adhes* 2012; **19**: 63-68 [PMID: 23181544 DOI: 10.3109/15419061.2012.748326]

113 **Bian Z**, Zhang J, Li M, Feng Y, Wang X, Zhang J, Yao S, Jin G, Du J, Han W, Yin Y, Huang S, Fei B, Zou J, Huang Z. LncRNA-FEZF1-AS1 Promotes Tumor Proliferation and Metastasis in Colorectal Cancer by Regulating PKM2 Signaling. *Clin Cancer Res* 2018; **24**: 4808-4819 [PMID: 29914894 DOI: 10.1158/1078-0432.CCR-17-2967]

114 **Xue J**, Liao L, Yin F, Kuang H, Zhou X, Wang Y. LncRNA AB073614 induces epithelial- mesenchymal transition of colorectal cancer cells via regulating the JAK/STAT3 pathway. *Cancer Biomark* 2018; **21**: 849-858 [PMID: 29439310 DOI: 10.3233/CBM-170780]

115 **Zhang Y**, Zhang K, Luo Z, Liu L, Wu L, Liu J. Circulating long non-coding HOX transcript antisense intergenic ribonucleic acid in plasma as a potential biomarker for diagnosis of breast cancer. *Thorac Cancer* 2016; **7**: 627-632 [PMID: 27755794 DOI: 10.1111/1759-7714.12373]

116 **Svoboda M**, Slyskova J, Schneiderova M, Makovicky P, Bielik L, Levy M, Lipska L, Hemmelova B, Kala Z, Protivankova M, Vycital O, Liska V, Schwarzova L, Vodickova L, Vodicka P. HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis* 2014; **35**: 1510-1515 [PMID: 24583926 DOI: 10.1093/carcin/bgu055]

117 **Li CY**, Liang GY, Yao WZ, Sui J, Shen X, Zhang YQ, Peng H, Hong WW, Ye YC, Zhang ZY, Zhang WH, Yin LH, Pu YP. Integrated analysis of long non-coding RNA competing interactions reveals the potential role in progression of human gastric cancer. *Int J Oncol* 2016; **48**: 1965-1976 [PMID: 26935047 DOI: 10.3892/ijo.2016.3407]

118 **Li P**, Zhang X, Wang L, Du L, Yang Y, Liu T, Li C, Wang C. lncRNA HOTAIR Contributes to 5FU Resistance through Suppressing miR-218 and Activating NF-κB/TS Signaling in Colorectal Cancer. *Mol Ther Nucleic Acids* 2017; **8**: 356-369 [PMID: 28918035 DOI: 10.1016/j.omtn.2017.07.007]

119 **Huang WW**, Hsieh KP, Huang RY, Yang YH. Role of cyclooxygenase-2 inhibitors in the survival outcome of colorectal cancer patients: A population-based cohort study. *Kaohsiung J Med Sci* 2017; **33**: 308-314 [PMID: 28601236 DOI: 10.1016/j.kjms.2017.03.004]

120 **Williams CS**, Shattuck-Brandt RL, DuBois RN. The role of COX-2 in intestinal cancer. *Expert Opin Investig Drugs* 1999; **8**: 1-12 [PMID: 15992053 DOI: 10.1517/13543784.8.1.1]

121 **Waddell WR**, Ganser GF, Cerise EJ, Loughry RW. Sulindac for polyposis of the colon. *Am J Surg* 1989; **157**: 175-179 [PMID: 2535920 DOI: 10.1016/0002-9610(89)90442-X]

122 **Rothwell PM**, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, Meade TW. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010; **376**: 1741-1750 [PMID: 20970847 DOI: 10.1016/S0140-6736(10)61543-7]

123 **Sandler RS**, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, Petrelli N, Pipas JM, Karp DD, Loprinzi CL, Steinbach G, Schilsky R. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* 2003; **348**: 883-890 [PMID: 12621132 DOI: 10.1056/NEJMoa021633]

124 **Ng K**, Meyerhardt JA, Chan AT, Sato K, Chan JA, Niedzwiecki D, Saltz LB, Mayer RJ, Benson AB, Schaefer PL, Whittom R, Hantel A, Goldberg RM, Venook AP, Ogino S, Giovannucci EL, Fuchs CS. Aspirin and COX-2 inhibitor use in patients with stage III colon cancer. *J Natl Cancer Inst* 2014; **107**: 345

[PMID: 25432409 DOI: 10.1093/jnci/dju345]
125 **Bos CL**, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, Peppelenbosch MP, Hardwick JC. Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. *Oncogene* 2006; **25**: 6447-6456 [PMID: 16878161 DOI: 10.1038/sj.onc.1209658]
126 **Burr NE**, Hull MA, Subramanian V. Does aspirin or non-aspirin non-steroidal anti-inflammatory drug use prevent colorectal cancer in inflammatory bowel disease? *World J Gastroenterol* 2016; **22**: 3679-3686 [PMID: 27053860 DOI: 10.3748/wjg.v22.i13.3679]
127 **Herfs M**, Hubert P, Delvenne P. Epithelial metaplasia: Adult stem cell reprogramming and (pre)neoplastic transformation mediated by inflammation? *Trends Mol Med* 2009; **15**: 245-253 [PMID: 19457719 DOI: 10.1016/j.molmed.2009.04.002]
128 **Liu XH**, Kirschenbaum A, Weinstein BM, Zaidi M, Yao S, Levine AC. Prostaglandin E2 modulates components of the Wnt signaling system in bone and prostate cancer cells. *Biochem Biophys Res Commun* 2010; **394**: 715-720 [PMID: 20227393 DOI: 10.1016/j.bbrc.2010.03.057]
129 **Tsoi KK**, Chan FC, Hirai HW, Sung JJ. Risk of gastrointestinal bleeding and benefit from colorectal cancer reduction from long-term use of low-dose aspirin: A retrospective study of 612 509 patients. *J Gastroenterol Hepatol* 2018; **33**: 1728-1736 [PMID: 29665624 DOI: 10.1111/jgh.14261]
130 **Biffi A**, Halpin A, Towfighi A, Gilson A, Busl K, Rost N, Smith EE, Greenberg MS, Rosand J, Viswanathan A. Aspirin and recurrent intracerebral hemorrhage in cerebral amyloid angiopathy. *Neurology* 2010; **75**: 693-698 [PMID: 20733144 DOI: 10.1212/WNL.0b013e3181eee40f]
131 **Grivennikov S**, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 2009; **15**: 103-113 [PMID: 19185845 DOI: 10.1016/j.ccr.2009.01.001]
132 **Peyrin-Biroulet L**. Anti-TNF therapy in inflammatory bowel diseases: A huge review. *Minerva Gastroenterol Dietol* 2010; **56**: 233-243 [PMID: 20485259]
133 **Ali T**, Kaitha S, Mahmood S, Ftesi A, Stone J, Bronze MS. Clinical use of anti-TNF therapy and increased risk of infections. *Drug Healthc Patient Saf* 2013; **5**: 79-99 [PMID: 23569399 DOI: 10.2147/DHPS.S28801]
134 **Hoshi D**, Nakajima A, Inoue E, Shidara K, Sato E, Kitahama M, Seto Y, Tanaka E, Urano W, Ichikawa N, Koseki Y, Momohara S, Taniguchi A, Nishimoto N, Yamanaka H. Incidence of serious respiratory infections in patients with rheumatoid arthritis treated with tocilizumab. *Mod Rheumatol* 2012; **22**: 122-127 [PMID: 21735355 DOI: 10.1007/s10165-011-0488-6]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2878-2886

DOI: 10.3748/wjg.v25.i23.2878

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

The role of endoscopy in the management of hereditary diffuse gastric cancer syndrome

Shria Kumar, Jessica M Long, Gregory G Ginsberg, Bryson W Katona

ORCID number: Shria Kumar (0000-0001-9745-2676); Jessica M Long (0000-0003-0382-7890); Gregory G Ginsberg (0000-0002-2023-7422); Bryson W Katona (0000-0001-8186-9119).

Author contributions: Kumar S and Katona BW planned the minireview format, reviewed literature, and wrote the paper; Long JM and Ginsberg GG provided expertise in genetics and endoscopy, respectively, and contributed via editing and writing

Supported by the National Institutes of Health T32 grant (Kumar S; Grant NO: 5T32DK7740-22).

Conflict-of-interest statement: Dr. Katona reports personal fees from Exact Sciences, other from Janssen, other from Immunovia, outside the submitted work.

Manuscript source: Invited manuscript

Shria Kumar, Gregory G Ginsberg, Bryson W Katona, Division of Gastroenterology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, United States

Jessica M Long, Division of Hematology and Oncology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, United States

Corresponding author: Bryson W Katona, MD, PhD, Division of Gastroenterology, University of Pennsylvania Perelman School of Medicine, 3400 Civic Center Blvd., 751 South Pavilion, Philadelphia, PA 19104, United States. bryson.katona@uphs.upenn.edu
Telephone: +1-215-349-8222
Fax: +1-215-349-5915

Abstract

Hereditary diffuse gastric cancer (HDGC) syndrome is an inherited cancer risk syndrome associated with pathogenic germline *CDH1* variants. Given the high risk for developing diffuse gastric cancer, *CDH1* carriers are recommended to undergo prophylactic total gastrectomy for cancer risk reduction. Current guidelines recommend upper endoscopy in *CDH1* carriers prior to surgery and then annually for individuals deferring prophylactic total gastrectomy. Management of individuals from HDGC families without *CDH1* pathogenic variants remains less clear, and management of families with *CDH1* pathogenic variants in the absence of a family history of gastric cancer is particularly problematic at present. Despite adherence to surveillance protocols, endoscopic detection of cancer foci in HDGC is suboptimal and imperfect for facilitating decision-making. Alternative endoscopic modalities, such as chromoendoscopy, endoscopic ultrasound, and other non-white light methods have been utilized, but are of limited utility to further improve cancer detection and risk stratification in HDGC. Herein, we review what is known and what remains unclear about endoscopic surveillance for HDGC, among individuals with and without germline *CDH1* pathogenic variants. Ultimately, the use of endoscopy in the management of HDGC remains a challenging arena, but one in which further research to improve surveillance is crucial.

Key words: *CDH1* gene; Hereditary diffuse gastric cancer; Gastric cancer; Endoscopic screening; Endoscopy

Core tip: Individuals with hereditary diffuse gastric cancer (HDGC) syndrome are at

Received: March 17, 2019
Peer-review started: March 19, 2019
First decision: April 4, 2019
Revised: April 8, 2019
Accepted: April 29, 2019
Article in press: April 29, 2019
Published online: June 21, 2019

P-Reviewer: Chen SQ, Cheng H, Xin Q
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

increased risk of diffuse gastric cancer, and are often recommended to undergo prophylactic total gastrectomy, especially in the presence of a pathogenic germline *CDH1* variant. Endoscopy is important in the initial management and surveillance of individuals with HDGC syndrome, yet sensitivity of endoscopy for detection of cancer foci in this population is poor. Alternative endoscopic modalities have not been found to be helpful. Ultimately, there is much to be learned about how to best use endoscopy in management of HDGC.

Citation: Kumar S, Long JM, Ginsberg GG, Katona BW. The role of endoscopy in the management of hereditary diffuse gastric cancer syndrome. *World J Gastroenterol* 2019; 25(23): 2878-2886
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2878.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2878

INTRODUCTION

Gastric cancer remains the fifth most common cancer worldwide. While the majority of cases are sporadic, 1-3% of gastric cancers are related to hereditary cancer syndromes, including hereditary diffuse gastric cancer syndrome (HDGC). Pa-thogenic germline *CDH1* variants have been associated with HDGC, although some families fulfilling HDGC clinical criteria do not have detectable germline variants[1]. *CDH1* encodes for the tumor suppressor E-cadherin, which serves as a critical cell adhesion molecule[2]. The connection of *CDH1* mutations to HDGC syndrome was first described in New Zealand, when Jones *et al*[1,3-5] suspected genetic predisposition as the cause of a high rate of gastric cancer in three Maori families. Since 1998, more than 120 different pathogenic variants of *CDH1* have been described, and carrying a germline *CDH1* pathogenic variant has been shown to portend a high risk of diffuse gastric cancer, characterized by signet ring cell carcinoma (SRCC) on histopathology, as well as lobular breast cancer in women[6-8].

Genetic testing for *CDH1* variants is recommended for those who meet clinical criteria for HDGC[1]. Criteria (considering first- and second-degree relatives) include having two or more family members with gastric cancer (including one confirmed diffuse gastric cancer), having one case of diffuse gastric cancer prior to age 40, or having both diffuse gastric cancer and lobular breast cancer in a family (with one diagnosed before age 50). Genetic testing can also be considered in those with cleft lip or palate and diffuse gastric cancer, in the presence of bilateral lobular breast cancer, or in families with two or more cases of lobular breast cancer before age 50. Testing for *CDH1* should include both sequencing and deletion/duplication analysis, and is now commonly performed by numerous commercial laboratories[9]. Germline *CDH1* pathogenic variants are found in approximately 25%-50% of families meeting HDGC criteria, though rates vary by ethnic background and country[9-12]. Those who meet testing criteria but do not have an identified *CDH1* pathogenic variant pose their own challenges in management and risk stra-tification. Recent studies have suggested *CTNNA1* and *MAP3K6* as other potential causative genes responsible for HDGC, however further work to confirm these associations is required[10,13]. Additionally, other cancer susceptibility genes associated with a spectrum of cancers outside of gastric cancer, such as *BRCA2*, *STK11*, and *PALB2* have also been identified in families meeting HDGC criteria, suggesting some clinically-defined HDGC families may have a genetic basis related to another hereditary syndrome[9].

The lifetime risk of diffuse gastric cancer in individuals with a germline *CDH1* pathogenic variant is reported to be up to 80%[1,14,15]. However, this high cumulative lifetime risk of diffuse gastric cancer may be an over-estimate, as the advent of multi-gene panel testing has identified a notable number of *CDH1* pathogenic variants in families without a history of diffuse gastric cancer[9,14-16], suggesting reduced penetrance in some families. Currently, individuals with a germline *CDH1* pathogenic variant are recommended to undergo prophylactic total gastrectomy, typically between the ages of 20-30[17,18]. However, given the major implications for quality of life and nutritional status after gastrectomy, especially in younger patients, some patients opt to delay or defer prophylactic total gastrectomy[19-22].

The role of endoscopy in the management of patients with HDGC has been studied extensively and plays an important role for diagnosis, surveillance, and risk stratification. Herein, we highlight the role of endoscopy in individuals with HDGC,

and review the recent research and advances in the field.

Guidelines for the use of endoscopy in HDGC with a known pathogenic CDH1 variant

For patients with a pathogenic germline *CDH1* variant who undergo prophylactic total gastrectomy, baseline upper endoscopy is recommended prior to surgery to evaluate for gross tumor or other concomitant pathology that may alter the surgical approach[1]. For those electing to defer risk reducing gastrectomy, annual surveillance endoscopy following the Cambridge protocol is advised. If microscopic or macroscopic disease is detected on surveillance, prompt gastrectomy referral is required[1,17]. White light exam is recommended for surveillance, since other endo-scopic modalities have not proven efficacious, as discussed below.

Surveillance endoscopy via the Cambridge protocol

The Cambridge protocol was developed to help guide endoscopic surveillance in HDGC[1]. Surveillance via the Cambridge protocol ideally should be performed in a high-volume center with a multi-disciplinary team having expertise in HDGC. Given the large number of biopsies performed, it is recommended to stop anticoagulation if possible prior to the procedure. Endoscopy should include a careful high definition white light (HDWL) exam in a session of at least 30 min. The endoscopic exam should include repeated insufflation and deflation to maximize visualization of the entire gastric mucosa and check for distensibility (the lack of which should raise concern for an infiltrative process such as *linitis plastica)*. Additionally, the exam should utilize extensive washing with the assistance of mucolytic and anti-foaming agents, to permit careful examination and documentation of the entire gastric mucosa. If there is concern for poor distensibility, further evaluation should include computed tomography scan and endoscopic ultrasonography (EUS).

Prior to obtaining random gastric biopsies, all visible lesions, pale areas (considered more likely to have abnormal signet ring cells), and other gastric abnormalities should be separately biopsied and sent for pathologic evaluation[5,23]. After sampling of all visible lesions, five random biopsies should then be taken from each of 6 anatomic regions: prepyloric, antrum, transitional zone, body, fundus, and cardia, with these groups of biopsies each being sent separately for pathologic analysis (see Figure 1). Despite no known association between *Helicobacter pylori* (*H. pylori*) and HDGC, baseline *H. pylori* testing on the gastric biopsy specimens is recommended given that *H. pylori* is considered a class I carcinogen by the World Health Organization. Subsequent treatment and confirmation of eradication of individuals who are *H. pylori* positive is advised[1,24].

EFFICACY OF ENDOSCOPY IN *CDH1* FAMILIES

Endoscopic detection of SRCC foci in *CDH1* carriers is poor, and evaluation of surgical pathology demonstrates cancer foci in 45%-60% of those with negative endoscopic evaluations[5,11,21,25]. The reason for this poor performance is due to the occult and difficult to predict nature of HDGC. HDGC is rarely restricted to one location in the stomach[6,26]. Independent studies have found vastly different results when evaluating the regions of the stomach where the HDGC-related SRCC foci are located[27-29]. This heterogeneity in location, as well as the inability to reliably visualize the SRCC foci on visual mucosal examination, both play a large role in the inability to reliably detect SRCC foci endoscopically. A model developed by Fujita *et al*[28] evaluated yield of cancer foci in a topographic pattern on 10 gastrectomy specimens from individuals with pathogenic germline *CDH1* variants. This model estimated that for a 90% detection of cancer foci, the theoretical number of biopsies necessary to capture at least a single cancer focus was estimated to be 1768 per patient, which is clearly not clinically feasible. However, the yield of endoscopically detecting SRCC foci preoperatively is both correlated with the number of biopsies taken and the number of SRCC lesions in the gastrectomy specimen[30]. As described by de Almeida *et al*[6], there are also practical issues beyond the number of biopsies required, including scarring from repeated biopsies, which may mimic pale areas concerning for malignancy and may cause confusion and repeated re-biopsy of the same areas of mucosa.

Unfortunately, the specific *CDH1* variant itself does not aid in determining the potential location of SRCC or the probability of finding SRCC, especially given the vast number of *CDH1* pathogenic variants that have been described[9,31,32]. There is also limited data of the benefit of continued surveillance in *CDH1* carriers. Most cancer foci are detected on the index endoscopy, which is likely due to detecting prevalent rather than incident cases[11]. This is likely due to the fact that multiple foci of SRCC

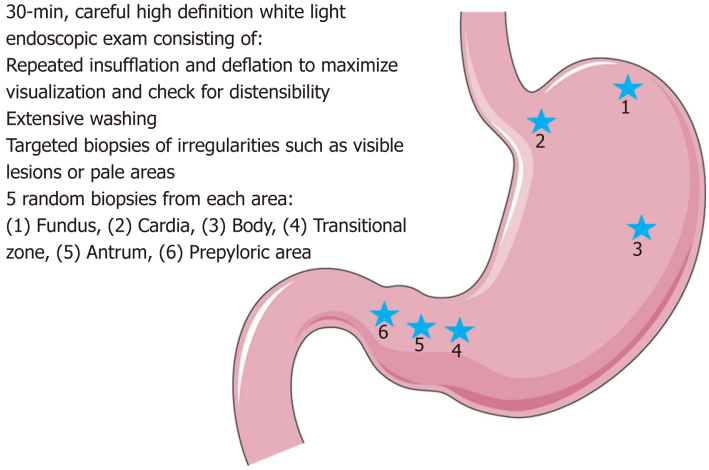


Figure 1 Illustrative representation of the Cambridge protocol for hereditary diffuse gastric cancer syndrome surveillance, full details in text and on image. Figure modified with text, markings (stars), and annotation after adaptation of "Stomach" from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License. Original photo adapted from https://smart.servier.com/smart_image/stomach-3/.

develop before age 30 and have a long indolent course[33]. It is further unknown how long these foci remain indolent, and what environmental or genetic predispositions can lead to more extensive disease.

In addition to the imperfect sensitivity of endoscopic surveillance, there are possible complications from endoscopy, as well as potential psychological hurdles in those individuals undergoing serial endoscopic evaluation, including concerns of lifelong endoscopy and the chances of false negatives[9,11,19,34]. The concern of false reassurance in those with a negative endoscopy is not to be underestimated, either.

Chromoendoscopy

In an attempt to improve the efficiency of SRCC detection in *CDH1* carriers, several advanced endoscopic techniques have been studied, including the use of chromoendoscopy. Based on a Japanese study, Shaw *et al*[5] published a series in 2005 describing annual chromoendoscopic surveillance in 33 patients with a pathogenic *CDH1* variant over a 5-year period. The patients initially drank a mucolytic (N-acetylcysteine) then changed position every 5 min for 30 min to ensure complete gastric coverage. The endoscopist then performed a HDWL endoscopic exam, and the patient received intravenous pentagastrin for gastric acid secretion, followed by spraying of methylene blue onto the mucosa. After absorption and suction, Congo red dye was sprayed and the mucosa was irrigated. Only pale areas were biopsied, and no random biopsies were performed. Of 56 total pale areas, 23 lesions (41%) showed SRCC (in 10 patients). When comparing these results to total gastrectomy pathology, it was shown that there was a positive correlation for foci 4-10 mm in size, but not for foci < 4 mm.

While initially promising, due to concerns about dye toxicity, chromoendoscopic exam is currently not recommended as standard of care for HDGC[1,6]. Additionally, there were further concerns regarding the learning curve in chromoendoscopy as well as pale area detection and interpretation, as the number of pale areas detected by endoscopists increased annually, despite no change in relative number of annual surveillance procedures[5,11].

Electronic enhanced imaging

Electronic enhanced imaging takes advantage of pre- and/or post-processing light wavelengths and filtering to amplify surface, subsurface and vascular prominence and contours. Beyond careful white-light examination with targeted and random biopsies, electronic enhanced imaging techniques have not proven to be particularly valuable for diagnosis, screening or surveillance of patients with pathologic *CDH1* gene mutations[5,25,35]. Lim *et al*[25] report that auto-fluorescence imaging (AFI) and narrow band imaging (NBI) were of limited utility, failing to reliably identify abnormal appearing mucosa for targeted tissue sampling. While AFI did not prove to aid in the detection of early foci of signet ring cell neoplasia, NBI was a useful imaging adjunct to delineate pale areas and, in conjunction with zoom magnification, allowed careful assessment of vascular and mucosal patterns, with a high negative predictive value in their study. Emerging electronic enhanced imaging techniques

with depth to the lamina propria layer will be tested in this condition owing to the unpredictable and patchy distribution of SRCC characteristic of early stage disease.

Endoscopic ultrasonography (EUS)

There is currently no recommendation for routine use of EUS in the management of HDGC. However, EUS can be considered for further investigation of abnormalities seen on standard HDWL endoscopic examination, such as poor distensibility of the stomach[1]. To evaluate the utility of EUS in patients with HDGC due to a *CDH1* pathogenic variant, a retrospective analysis of 13 patients who underwent radial scanning endosonography in addition to guideline-recommended upper endoscopy before gastrectomy found no benefit in performing endoscopic ultrasound to improve detection of cancer foci[34]. In that study, the sensitivity for identifying SRCC foci by HDWL endoscopy was 45%, similar to other descriptive series.

Confocal endoscopic microscopy

There is an ongoing NIH clinical trial evaluating the use of confocal endoscopic microscopy, which provides histologic imaging of gastric mucosa during upper endoscopy, and could improve endoscopic surveillance sensitivity (ClinicalTrials.gov Identifier: NCT03648879)[36]. However, this technique remains experimental at this time, and should only be pursued in HDGC through a clinical trial.

Colorectal cancer screening

Although the majority of research on HDGC has focused on the role of upper endoscopic examination, there is limited evidence that there may be an association of colorectal SRCC in patients with germline *CDH1* pathogenic variants[1]. More recent work has also speculated that the *CDH1* variant location may be an important predictor of colorectal cancer risk[37]. Although based on limited data, enhanced colorectal cancer screening by colonoscopy is recommended for *CDH1* carriers with a family history of colon cancer, especially when there is a presence of signet ring cells and/or mucinous features of the cancer[1,17]. In these individuals, screening should start at age 40 or 10 years prior to the youngest diagnosis in the family, and repeated every 3-5 years. In all other *CDH1* carriers, standard colorectal cancer screening guidelines should be followed based on the individual's personal and/or family history.

ROLE OF ENDOSCOPY IN PATIENTS WITH A PATHOGENIC *CDH1* VARIANT AND NO FAMILY HISTORY OF GASTRIC CANCER

Individuals with a pathogenic variant of *CDH1*, but without family history of gastric cancer, pose a unique challenge. There is limited evidence on endoscopic surveillance of these patients, and one small series found that despite no family history of gastric cancer and negative endoscopic evaluation, 50% of patients had SRCC on surgical pathology[38]. It remains to be determined if these patients truly have decreased gastric cancer risk and reduced prevalence compared to *CDH1* carriers with a strong family history of gastric cancer[9,14-16]. Another possibility is that in small or poorly reported-on families, family history may be inaccurate, as even in the best circumstances, family history collection can be poor[39-41]. As such, these patients may have a true family history of gastric cancer that simply goes unrecognized.

A similar dilemma arises in *CDH1* carriers with lobular breast cancer and without a family history of diffuse gastric cancer. Recent studies regarding the management of lobular breast cancer, to which a pathogenic variant of *CDH1* increased risk, in the absence of gastric cancer family history have been small, though informative[42-46]. These studies demonstrate that though rare, even in the absence of familial gastric cancer, lobular breast cancer may develop, and may even represent a separate cancer syndrome[44,45,47]. This has clinical consequences for the screening of breast cancer in those with a pathogenic variant of *CDH1*, though the need for gastrectomy in these individuals is similarly cloudy[42,43,47]. There have not been large reports of endoscopic utility in these patients.

An advantage of endoscopic surveillance in these groups would be the finding of SRCC on endoscopic biopsy, as this would justify the need for gastrectomy in the physician and patient alike. However, we would expect the sensitivity of endoscopy to be lacking, as it is in those with pathogenic variants of *CDH1* and HDGC, and therefore a negative endoscopic examination should not be overly reassuring regarding gastric cancer risk. This is an area with much uncertainty that requires further research, especially in light of the growing preponderance of multi-gene panel

testing with its resulting identification of incidental *CDH1* variants in families without a history of *CDH1*-associated cancers.

ROLE OF ENDOSCOPY IN HDGC FAMILIES WITHOUT A PATHOGENIC *CDH1* VARIANT

Individuals from families meeting HDGC criteria who lack a germline *CDH1* pathogenic variant present a significant challenge as prophylactic total gastrectomy is typically not recommended for these individuals. In these cases, endoscopic surveillance is recommended on a regular basis in a high-volume specialty center, as the identification of SRCC foci on HDWL endoscopy in these individuals can be helpful for guiding future surgical management[1].

However, the yield of endoscopy for identifying malignant lesions is lower in those from families meeting HDGC criteria without a detectable *CDH1* pathogenic variant. This was demonstrated in a United Kingdom-based prospective cohort study of endoscopic surveillance in families with and without *CDH1* pathogenic variants; patients with *CDH1* pathogenic variants had significantly higher rates of SRCC on endoscopy than their *CDH1* negative counterparts[11]. In this study, 85 individuals underwent endoscopic surveillance, including 54 patients (63.5%) with a *CDH1* pathogenic variant opting to delay gastrectomy and 31 patients undergoing surveillance who were *CDH1* negative. Endoscopic surveillance included 30 random biopsies as well as targeted biopsy of mucosal abnormalities. Of those with *CDH1* pathogenic variants, 33 (61.1%) had foci of SRCC detected during surveillance, the majority of which occurred at the index endoscopy. Those without a *CDH1* pathogenic variant had a much lower detection rate of SRCC, 3 of 31 (9.7%) patients. This difference was statistically significant ($P < 0.0005$), and no other factors, including age, sex, proton pump inhibitor use, or *H. pylori* infection were associated with the differential detection of cancer foci.

It has been considered that there may be a different pathogenesis for diffuse gastric cancer in *CDH1*-negative HDGC individuals, *i.e.*, that SRCC may not be the preceding lesion; however, this remains unclear and further investigation is required[11]. Given the number of unanswered questions, including pathogenesis, yield of endoscopy, and rate and frequency of progression to cancer, the management of individuals from *CDH1*-negative HDGC families remains challenging. Mi *et al*[11] suggested that risk stratification using allelic expression imbalance (as a marker for progression to neoplasia) may be helpful to better determine the risk for these individuals. Ideally in the future, enhanced understanding of the genetic basis for HDGC families currently without detectable *CDH1* pathogenic variants (such as via improved *CDH1* variant detection or identification of additional gastric cancer risk genes) may enable better risk stratification in this population.

Currently, endoscopic surveillance at a referral center is suggested for individuals from families meeting HDGC criteria and who lack a *CDH1* pathogenic variant. It is clear in this high-risk group that endoscopic detection of a gastric malignancy, including foci of SRCC, should prompt a referral for consideration of total gastrectomy, yet counseling these patients on negative endoscopies presents its own challenge. Additionally, the optimal frequency of surveillance in this population also remains to be determined[18].

CONCLUSION

Management of patients with HDGC, both with and without pathogenic variants in *CDH1*, remains challenging given our lack of understanding about the critical drivers of penetrance, differential anatomical location of SRCC, and the imperfect sensitivity of endoscopy to detect early foci of SRCC. However, given the uncertainties in the field, the use of endoscopy in these individuals is an area that is ripe for future research, as improvement in endoscopic surveillance in HDGC would undoubtedly improve risk stratification, surgical management, and overall patient well-being in families with HDGC.

REFERENCES

1 **van der Post RS**, Vogelaar IP, Carneiro F, Guilford P, Huntsman D, Hoogerbrugge N, Caldas C, Schreiber KE, Hardwick RH, Ausems MG, Bardram L, Benusiglio PR, Bisseling TM, Blair V, Bleiker E, Boussioutas A, Cats A, Coit D, DeGregorio L, Figueiredo J, Ford JM, Heijkoop E, Hermens R, Humar B,

Kaurah P, Keller G, Lai J, Ligtenberg MJ, O'Donovan M, Oliveira C, Pinheiro H, Ragunath K, Rasenberg E, Richardson S, Roviello F, Schackert H, Seruca R, Taylor A, Ter Huurne A, Tischkowitz M, Joe ST, van Dijck B, van Grieken NC, van Hillegersberg R, van Sandick JW, Vehof R, van Krieken JH, Fitzgerald RC. Hereditary diffuse gastric cancer: Updated clinical guidelines with an emphasis on germline CDH1 mutation carriers. *J Med Genet* 2015; **52**: 361-374 [PMID: 25979631 DOI: 10.1136/jmedgenet-2015-103094]

2 **Pećina-Slaus N**. Tumor suppressor gene E-cadherin and its role in normal and malignant cells. *Cancer Cell Int* 2003; **3**: 17 [PMID: 14613514 DOI: 10.1186/1475-2867-3-17]

3 **van der Post RS**, Carneiro F. Emerging Concepts in Gastric Neoplasia: Heritable Gastric Cancers and Polyposis Disorders. *Surg Pathol Clin* 2017; **10**: 931-945 [PMID: 29103540 DOI: 10.1016/j.path.2017.07.011]

4 **Jones EG**. Familial Gastric Cancer. *N Z Med J* 1964; **63**: 287-296 [PMID: 14158754]

5 **Shaw D**, Blair V, Framp A, Harawira P, McLeod M, Guilford P, Parry S, Charlton A, Martin I. Chromoendoscopic surveillance in hereditary diffuse gastric cancer: An alternative to prophylactic gastrectomy? *Gut* 2005; **54**: 461-468 [PMID: 15753528 DOI: 10.1136/gut.2004.049171]

6 **de Almeida Artifon EL**, Marinho FRT. Endoscopic screening for hereditary diffuse gastric cancer: One size does not fit all. *Gastrointest Endosc* 2018; **87**: 405-407 [PMID: 29406927 DOI: 10.1016/j.gie.2017.05.042]

7 **Katona BW**, Rustgi AK. Gastric Cancer Genomics: Advances and Future Directions. *Cell Mol Gastroenterol Hepatol* 2017; **3**: 211-217 [PMID: 28275688 DOI: 10.1016/j.jcmgh.2017.01.003]

8 **Guilford P**, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998; **392**: 402-405 [PMID: 9537325 DOI: 10.1038/32918]

9 **Hansford S**, Kaurah P, Li-Chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S, Lum A, Talhouk A, Baker-Lange K, Richardson S, Lewis I, Lindor NM, Pennell E, MacMillan A, Fernandez B, Keller G, Lynch H, Shah SP, Guilford P, Gallinger S, Corso G, Roviello F, Caldas C, Oliveira C, Pharoah PD, Huntsman DG. Hereditary Diffuse Gastric Cancer Syndrome: CDH1 Mutations and Beyond. *JAMA Oncol* 2015; **1**: 23-32 [PMID: 26182300 DOI: 10.1001/jamaoncol.2014.168]

10 **Gaston D**, Hansford S, Oliveira C, Nightingale M, Pinheiro H, Macgillivray C, Kaurah P, Rideout AL, Steele P, Soares G, Huang WY, Whitehouse S, Blowers S, LeBlanc MA, Jiang H, Greer W, Samuels ME, Orr A, Fernandez CV, Majewski J, Ludman M, Dyack S, Penney LS, McMaster CR, Huntsman D, Bedard K. Germline mutations in MAP3K6 are associated with familial gastric cancer. *PLoS Genet* 2014; **10**: e1004669 [PMID: 25340522 DOI: 10.1371/journal.pgen.1004669]

11 **Mi EZ**, Mi EZ, di Pietro M, O'Donovan M, Hardwick RH, Richardson S, Ziauddeen H, Fletcher PC, Caldas C, Tischkowitz M, Ragunath K, Fitzgerald RC. Comparative study of endoscopic surveillance in hereditary diffuse gastric cancer according to CDH1 mutation status. *Gastrointest Endosc* 2018; **87**: 408-418 [PMID: 28688938 DOI: 10.1016/j.gie.2017.06.028]

12 **Sugimoto S**, Komatsu H, Morohoshi Y, Kanai T. Recognition of and recent issues in hereditary diffuse gastric cancer. *J Gastroenterol* 2015; **50**: 831-843 [PMID: 26049741 DOI: 10.1007/s00535-015-1093-9]

13 **Weren RDA**, van der Post RS, Vogelaar IP, van Krieken JH, Spruijt L, Lubinski J, Jakubowska A, Teodorczyk U, Aalfs CM, van Hest LP, Oliveira C, Kamping EJ, Schackert HK, Ranzani GN, Gómez García EB, Hes FJ, Holinski-Feder E, Genuardi M, Ausems MGEM, Sijmons RH, Wagner A, van der Kolk LE, Cats A, Bjørnevoll I, Hoogerbrugge N, Ligtenberg MJL. Role of germline aberrations affecting CTNNA1, MAP3K6 and MYD88 in gastric cancer susceptibility. *J Med Genet* 2018; **55**: 669-674 [PMID: 29330337 DOI: 10.1136/jmedgenet-2017-104962]

14 **Gullo I**, Devezas V, Baptista M, Garrido L, Castedo S, Morais R, Wen X, Rios E, Pinheiro J, Pinto-Ribeiro I, Ferreira RM, Preto J, Santos-Antunes J, Marques M, Campos M, Almeida F, Espinheira MDC, Amil Dias J, Figueiredo C, Oliveira C, Trindade E, Carneiro F. Phenotypic heterogeneity of hereditary diffuse gastric cancer: Report of a family with early-onset disease. *Gastrointest Endosc* 2018; **87**: 1566-1575 [PMID: 29454568 DOI: 10.1016/j.gie.2018.02.008]

15 **Huntsman DG**, Carneiro F, Lewis FR, MacLeod PM, Hayashi A, Monaghan KG, Maung R, Seruca R, Jackson CE, Caldas C. Early gastric cancer in young, asymptomatic carriers of germ-line E-cadherin mutations. *N Engl J Med* 2001; **344**: 1904-1909 [PMID: 11419427 DOI: 10.1056/NEJM200106213442504]

16 **Huynh JM**, Laukaitis CM. Panel testing reveals nonsense and missense CDH1 mutations in families without hereditary diffuse gastric cancer. *Mol Genet Genomic Med* 2016; **4**: 232-236 [PMID: 27064202 DOI: 10.1002/mgg3.197]

17 **Syngal S**, Brand RE, Church JM, Giardiello FM, Hampel HL, Burt RW; American College of Gastroenterology. ACG clinical guideline: Genetic testing and management of hereditary gastrointestinal cancer syndromes. *Am J Gastroenterol* 2015; **110**: 223-62; quiz 263 [PMID: 25645574 DOI: 10.1038/ajg.2014.435]

18 **van der Post RS**, van Dieren J, Grelack A, Hoogerbrugge N, van der Kolk LE, Snaebjornsson P, Lansdorp-Vogelaar I, van Krieken JH, Bisseling TM, Cats A. Outcomes of screening gastroscopy in first-degree relatives of patients fulfilling hereditary diffuse gastric cancer criteria. *Gastrointest Endosc* 2018; **87**: 397-404.e2 [PMID: 28455161 DOI: 10.1016/j.gie.2017.04.016]

19 **Hallowell N**, Badger S, Richardson S, Caldas C, Hardwick RH, Fitzgerald RC, Lawton J. An investigation of the factors effecting high-risk individuals' decision-making about prophylactic total gastrectomy and surveillance for hereditary diffuse gastric cancer (HDGC). *Fam Cancer* 2016; **15**: 665-676 [PMID: 27256430 DOI: 10.1007/s10689-016-9910-8]

20 **Hallowell N**, Lawton J, Badger S, Richardson S, Hardwick RH, Caldas C, Fitzgerald RC. The Psychosocial Impact of Undergoing Prophylactic Total Gastrectomy (PTG) to Manage the Risk of Hereditary Diffuse Gastric Cancer (HDGC). *J Genet Couns* 2017; **26**: 752-762 [PMID: 27837291 DOI: 10.1007/s10897-016-0045-8]

21 **Moslim MA**, Heald B, Tu C, Burke CA, Walsh RM. Early genetic counseling and detection of CDH1 mutation in asymptomatic carriers improves survival in hereditary diffuse gastric cancer. *Surgery* 2018; **164**: 754-759 [PMID: 30145018 DOI: 10.1016/j.surg.2018.05.059]

22 **Worster E**, Liu X, Richardson S, Hardwick RH, Dwerryhouse S, Caldas C, Fitzgerald RC. The impact of prophylactic total gastrectomy on health-related quality of life: A prospective cohort study. *Ann Surg* 2014; **260**: 87-93 [PMID: 24424140 DOI: 10.1097/SLA.0000000000000446]

23 **Fitzgerald RC**, Hardwick R, Huntsman D, Carneiro F, Guilford P, Blair V, Chung DC, Norton J,

Ragunath K, Van Krieken JH, Dwerryhouse S, Caldas C; International Gastric Cancer Linkage Consortium. Hereditary diffuse gastric cancer: Updated consensus guidelines for clinical management and directions for future research. *J Med Genet* 2010; **47**: 436-444 [PMID: 20591882 DOI: 10.1136/jmg.2009.074237]

24 **Vogiatzi P**, Cassone M, Luzzi I, Lucchetti C, Otvos L, Giordano A. Helicobacter pylori as a class I carcinogen: Physiopathology and management strategies. *J Cell Biochem* 2007; **102**: 264-273 [PMID: 17486575 DOI: 10.1002/jcb.21375]

25 **Lim YC**, di Pietro M, O'Donovan M, Richardson S, Debiram I, Dwerryhouse S, Hardwick RH, Tischkowitz M, Caldas C, Ragunath K, Fitzgerald RC. Prospective cohort study assessing outcomes of patients from families fulfilling criteria for hereditary diffuse gastric cancer undergoing endoscopic surveillance. *Gastrointest Endosc* 2014; **80**: 78-87 [PMID: 24472763 DOI: 10.1016/j.gie.2013.11.040]

26 **Carneiro F**, Huntsman DG, Smyrk TC, Owen DA, Seruca R, Pharoah P, Caldas C, Sobrinho-Simões M. Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening. *J Pathol* 2004; **203**: 681-687 [PMID: 15141383 DOI: 10.1002/path.1564]

27 **Charlton A**, Blair V, Shaw D, Parry S, Guilford P, Martin IG. Hereditary diffuse gastric cancer: Predominance of multiple foci of signet ring cell carcinoma in distal stomach and transitional zone. *Gut* 2004; **53**: 814-820 [PMID: 15138207 DOI: 10.1016/S0016-5085(03)84094-5]

28 **Fujita H**, Lennerz JK, Chung DC, Patel D, Deshpande V, Yoon SS, Lauwers GY. Endoscopic surveillance of patients with hereditary diffuse gastric cancer: Biopsy recommendations after topographic distribution of cancer foci in a series of 10 CDH1-mutated gastrectomies. *Am J Surg Pathol* 2012; **36**: 1709-1717 [PMID: 23073328 DOI: 10.1097/PAS.0b013e31826ca204]

29 **Rogers WM**, Dobo E, Norton JA, Van Dam J, Jeffrey RB, Huntsman DG, Kingham K, Chun N, Ford JM, Longacre TA. Risk-reducing total gastrectomy for germline mutations in E-cadherin (CDH1): Pathologic findings with clinical implications. *Am J Surg Pathol* 2008; **32**: 799-809 [PMID: 18391748 DOI: 10.1097/PAS.0b013e31815e7f1a]

30 **Barber ME**, Save V, Carneiro F, Dwerryhouse S, Lao-Sirieix P, Hardwick RH, Caldas C, Fitzgerald RC. Histopathological and molecular analysis of gastrectomy specimens from hereditary diffuse gastric cancer patients has implications for endoscopic surveillance of individuals at risk. *J Pathol* 2008; **216**: 286-294 [PMID: 18825658 DOI: 10.1002/path.2415]

31 **Guilford P**, Humar B, Blair V. Hereditary diffuse gastric cancer: Translation of CDH1 germline mutations into clinical practice. *Gastric Cancer* 2010; **13**: 1-10 [PMID: 20373070 DOI: 10.1007/s10120-009-0531-x]

32 **Luo W**, Fedda F, Lynch P, Tan D. CDH1 Gene and Hereditary Diffuse Gastric Cancer Syndrome: Molecular and Histological Alterations and Implications for Diagnosis And Treatment. *Front Pharmacol* 2018; **9**: 1421 [PMID: 30568591 DOI: 10.3389/fphar.2018.01421]

33 **Tan RY**, Ngeow J. Hereditary diffuse gastric cancer: What the clinician should know. *World J Gastrointest Oncol* 2015; **7**: 153-160 [PMID: 26380059 DOI: 10.4251/wjgo.v7.i9.153]

34 **Kumar S**, Katona BW, Long JM, Domchek S, Rustgi AK, Roses R, Ginsberg GG. Endoscopic Ultrasound Has Limited Utility in Diagnosis of Gastric Cancer in Carriers of CDH1 Mutations. *Clin Gastroenterol Hepatol* 2019 [PMID: 31077828 DOI: 10.1016/j.cgh.2019.04.064]

35 **Brandão C**, Dinis-Ribeiro M. Early diagnosis of hereditary diffuse gastric cancer: (Not only) an endoscopic challenge! *Endosc Int Open* 2016; **4**: E1311-E1312 [PMID: 27995194 DOI: 10.1055/s-0042-121003]

36 **Templeton A**, Hwang JH. Confocal microscopy in the esophagus and stomach. *Clin Endosc* 2013; **46**: 445-449 [PMID: 24143300 DOI: 10.5946/ce.2013.46.5.445]

37 **Lo W**, Zhu B, Sabesan A, Wu HH, Powers A, Sorber RA, Ravichandran S, Chen I, McDuffie LA, Quadri HS, Beane JD, Calzone K, Miettinen MM, Hewitt SM, Koh C, Heller T, Wacholder S, Rudloff U. Associations of CDH1 germline variant location and cancer phenotype in families with hereditary diffuse gastric cancer (HDGC). *J Med Genet* 2019; **pii**: jmedgenet-2018-105361 [PMID: 30745422 DOI: 10.1136/jmedgenet-2018-105361]

38 **Stoffel EM**, Koeppe E, Hanson K, Osborne J, Jacobs M, Appelman HD, Wong S. Sa1765 Incidental Finding of Germline CDH1 Mutations: Implications for Clinical Management. *Gastroenterology* 2016; **150**: S360 [DOI: 10.1016/S0016-5085(16)31265-3]

39 **Mai PL**, Garceau AO, Graubard BI, Dunn M, McNeel TS, Gonsalves L, Gail MH, Greene MH, Willis GB, Wideroff L. Confirmation of family cancer history reported in a population-based survey. *J Natl Cancer Inst* 2011; **103**: 788-797 [PMID: 21562245 DOI: 10.1093/jnci/djr114]

40 **Murff HJ**, Spigel DR, Syngal S. Does this patient have a family history of cancer? An evidence-based analysis of the accuracy of family cancer history. *JAMA* 2004; **292**: 1480-1489 [PMID: 15383520 DOI: 10.1001/jama.292.12.1480]

41 **Fiederling J**, Shams AZ, Haug U. Validity of self-reported family history of cancer: A systematic literature review on selected cancers. *Int J Cancer* 2016; **139**: 1449-1460 [PMID: 27222437 DOI: 10.1002/ijc.30203]

42 **Benusiglio PR**, Malka D, Rouleau E, De Pauw A, Buecher B, Noguès C, Fourme E, Colas C, Coulet F, Warcoin M, Grandjouan S, Sezeur A, Laurent-Puig P, Molière D, Tlemsani C, Di Maria M, Byrde V, Delaloge S, Blayau M, Caron O. CDH1 germline mutations and the hereditary diffuse gastric and lobular breast cancer syndrome: A multicentre study. *J Med Genet* 2013; **50**: 486-489 [PMID: 23709761 DOI: 10.1136/jmedgenet-2012-101472]

43 **Masciari S**, Larsson N, Senz J, Boyd N, Kaurah P, Kandel MJ, Harris LN, Pinheiro HC, Troussard A, Miron P, Tung N, Oliveira C, Collins L, Schnitt S, Garber JE, Huntsman D. Germline E-cadherin mutations in familial lobular breast cancer. *J Med Genet* 2007; **44**: 726-731 [PMID: 17660459 DOI: 10.1136/jmg.2007.051268]

44 **Schrader KA**, Masciari S, Boyd N, Salamanca C, Senz J, Saunders DN, Yorida E, Maines-Bandiera S, Kaurah P, Tung N, Robson ME, Ryan PD, Olopade OI, Domchek SM, Ford J, Isaacs C, Brown P, Balmana J, Razzak AR, Miron P, Coffey K, Terry MB, John EM, Andrulis IL, Knight JA, O'Malley FP, Daly M, Bender P, kConFab, Moore R, Southey MC, Hopper JL, Garber JE, Huntsman DG. Germline mutations in CDH1 are infrequent in women with early-onset or familial lobular breast cancers. *J Med Genet* 2011; **48**: 64-68 [PMID: 20921021 DOI: 10.1136/jmg.2010.079814]

45 **Xie ZM**, Li LS, Laquet C, Penault-Llorca F, Uhrhammer N, Xie XM, Bignon YJ. Germline mutations of the E-cadherin gene in families with inherited invasive lobular breast carcinoma but no diffuse gastric cancer. *Cancer* 2011; **117**: 3112-3117 [PMID: 21271559 DOI: 10.1002/cncr.25876]

46 **Petridis C**, Shinomiya I, Kohut K, Gorman P, Caneppele M, Shah V, Troy M, Pinder SE, Hanby A,

Tomlinson I, Trembath RC, Roylance R, Simpson MA, Sawyer EJ. Germline CDH1 mutations in bilateral lobular carcinoma in situ. *Br J Cancer* 2014; **110**: 1053-1057 [PMID: 24366306 DOI: 10.1038/bjc.2013.792]

47 **Corso G**, Intra M, Trentin C, Veronesi P, Galimberti V. CDH1 germline mutations and hereditary lobular breast cancer. *Fam Cancer* 2016; **15**: 215-219 [PMID: 26759166 DOI: 10.1007/s10689-016-9869-5]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2887-2897

DOI: 10.3748/wjg.v25.i23.2887

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Predicting systemic spread in early colorectal cancer: Can we do better?

Scarlet Fiona Brockmoeller, Nicholas Paul West

ORCID number: Scarlet Fiona Brockmoeller (0000-0002-7452-5833); Nicholas Paul West (0000-0002-0346-6709).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Supported by Yorkshire Cancer Research; and the National Institute for Health Research, the Pelican Cancer Foundation and the Pathological Society of Great Britain and Ireland.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Received: March 20, 2019
Peer-review started: March 20, 2019
First decision: April 4, 2019
Revised: May 7, 2019

Scarlet Fiona Brockmoeller, Nicholas Paul West, Pathology and Data Analytics, Leeds Institute of Medical Research at St. James's, University of Leeds, School of Medicine, Leeds LS9 7TF, United Kingdom

Corresponding author: Nicholas Paul West, BSc, MBChB, PhD, Doctor, Senior Lecturer, Pathology and Data Analytics, University of Leeds, Level 4 Wellcome Trust Brenner building, St. James's University Hospital, Beckett Street, Leeds LS9 7TF, United Kingdom.
n.p.west@leeds.ac.uk
Telephone: +44-113-3438626

Abstract

Through the implementation of national bowel cancer screening programmes we have seen a three-fold increase in early pT1 colorectal cancers, but how these lesions should be managed is currently unclear. Local excision can be an attractive option, especially for fragile patients with multiple comorbidities, but it is only safe from an oncological point of view in the absence of lymph node metastasis. Patient risk stratification through careful analysis of histopathological features in local excision or polypectomy specimens should be performed according to national guidelines to avoid under- or over-treatment. Currently national guidelines vary in their recommendations as to which factors should be routinely reported and there is no established multivariate risk stratification model to determine which patients should be offered major resectional surgery. Conventional histopathological parameters such as tumour grading or lymphovascular invasion have been shown to be predictive of lymph node metastasis in a number of studies but the inter- and intra-observer variation in reporting is high. Newer parameters including tumour budding and poorly differentiated clusters have been shown to have great potential, but again some improvement in the inter-observer variation is required. With the implementation of digital pathology into clinical practice, quantitative parameters like depth/area of submucosal invasion and proportion of stroma can be routinely assessed. In this review we present the various histopathological risk factors for predicting systemic spread in pT1 colorectal cancer and introduce potential novel quantitative variables and multivariable risk models that could be used to better define the optimal treatment of this increasingly common disease.

Key words: Early colorectal cancer; Bowel cancer screening; Local resection; Major resection; Morphological risk factors; Conventional histopathology parameters; Novel histopathology parameters; Risk stratification models; Digital pathology

Accepted: May 8, 2019
Article in press: May 8, 2019
Published online: June 21, 2019

P-Reviewer: Merkel S, Tandon RK
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

Core tip: Since the implementation of national bowel cancer screening programmes we have seen a three-fold increase in early colorectal cancers but how these lesion should surgical managed is currently unclear. Conventional histopathological parameters such as tumour grading or lymphovascular invasion have been shown to be predictive of lymph node metastasis but the inter- and intra-observer variation in reporting is significant. This review present the various conventional histopathological risk factors for predicting systemic spread in pT1 colorectal cancer and introduces novel quantitative variables and multivariable risk models that could be used to better define the optimal treatment of this increasingly common disease.

Citation: Brockmoeller SF, West NP. Predicting systemic spread in early colorectal cancer: Can we do better? *World J Gastroenterol* 2019; 25(23): 2887-2897
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2887.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2887

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers worldwide[1,2]. It is the third most common cause of cancer death in the United Kingdom and the United States in both females and males[1]. Through the implementation of national population screening[3] like the United Kingdom National Health Service Bowel Cancer Screening Programme (NHS BCSP) we have observed a three-fold increase in early CRC (stage pT1) from 5% to 17%[4]. Early CRC is defined as the "invasion of neoplastic glandular epithelial cells through the muscularis mucosae into the submucosa of the bowel wall but not beyond"[5].

Currently it is unclear as to how pT1 CRC should be optimally managed. Major bowel resection can be performed, however, this is associated with a significant risk of post-operative mortality, especially in elderly patients, and also morbidity including permanent colostomy formation, sexual and genitourinary problems, and low anterior resection syndrome. Internationally, postoperative mortality rates vary markedly, largely depending on background comorbidity in the population. Local excision of the tumour and avoidance of major surgery is an attractive option for patients with rectal cancer or significant comorbidity, but this is only safe from an oncological viewpoint in the absence of lymph node metastasis (LNM)[6,7]. It is therefore important that when deciding whether major bowel surgery or local excision should be performed, the postoperative mortality and lymph node metastasis risk are accurately estimated to inform the decision. Approximately 10%-15% of all pT1 CRC have LNM at the time of primary diagnosis with pedunculated pT1 CRC having an even lower risk (3% to 7% in the Asian population). Despite this major bowel resection rates in pT1 CRC can be as high as 76%, meaning that many patients are potentially exposed to unnecessary risk[8-11].

When a local excision (including polypectomy) is performed for pT1 CRC, patient risk stratification is undertaken by histopathologists through careful analysis of the specimen to determine the risk of LNM. The detailed macroscopic and microscopic evaluation of the specimen produces a large amount of information to guide further treatment. Routine information that is generally collected internationally includes the type of tumour, differentiation grade, TNM stage, level of invasion, number of lymph nodes involved, lymphatic invasion status, venous invasion status, perineural invasion status and resection margin status[12]. Currently various national guidelines differ in their recommendations as to which histopathological factors should be reported and used to determine the risk of LNM and therefore use of major surgery, and there is no established multivariate risk stratification model. The classification of some factors is also not performed according to an international standard with various systems in use. This review will present and discuss the various histopathological risk factors that can be used to predict systemic spread in in pT1 CRC and introduce potential novel quantitative variables and multivariable risk models that could be used to better define the optimal treatment of this increasingly common disease.

LITERATURE SEARCH

Literature searching was performed in PubMed (https://www.ncbi.nlm.nih.gov/pubmed) for the following keywords: "lymph nodes", "lymph node metastasis", "T1" and "pT1" combined with "colorectal cancer". Articles published between July 2004 and January 2019 were reviewed. In addition, manual cross-referencing was performed and further relevant papers published before 2007 identified through review articles. Inclusion criteria for studies included publication in English, use of at least 100 patients and availability of LNM status. Studies were excluded if neo-adjuvant therapy was used due to the potential effect on tumour staging.

CONVENTIONAL HISTOPATHOLOGICAL PARAMETERS

Histological tumour type

The majority of CRCs are adenocarcinoma but specific histological variants including cribriform or micropapillary adenocarcinoma have been reported in case series to have a higher rate of LNM in early CRC[13], and may be used to indicate further treatment in margin negative local excisions[14]. Mucinous adenocarcinomas (> 50% of the tumour area composed of extracellular mucin), signet ring cell adenocarcinomas (> 50% of the area composed of signet ring cells) and medullary carcinomas are associated with deficient mismatch repair (dMMR), which has a better prognosis when compared to cases with proficient mismatch repair. Yet in the absence of deficient mismatch repair, these histological subtypes are associated with a poorer prognosis than conventional CRC and further treatment may be indicated. The importance of routine mismatch repair immunostaining is detailed below.

Tumour differentiation grading

Poor tumour differentiation has been shown in numerous studies, including meta-analyses, to be significantly associated with poorer survival[15-17] and prediction of LNM in early CRC[7,18]. Poor differentiation is primarily based on the architecture of the tumour and the hallmark is the absence of any tubular formation or irregularly folded, distorted and often small tubules. Tumour differentiation is a subjective parameter and grading may vary between assessors. Currently the WHO grading system proposes a four tier classification including: Grade 1 (well differentiated), grade 2 (moderately differentiated), grade 3 (poorly differentiated) and grade 4 (undifferentiated)[5]. An improvement in inter-observer agreement can be achieved through compressing this system into two grades, *i.e.*, well/moderate differentiation *vs* poor differentiation[19]. This has been routinely adopted in the United Kingdom on the basis that poor differentiation is an important high-risk feature in early CRC. With the implementation of digital pathology into routine clinical practice, tumour differentiation grading could be further improved by the implementation of automated algorithms to reduce the subjectivity of pathologist assessment[20-22].

For local excision specimens, there is currently uncertainty as to whether grading should be based on the predominant or worst area of differentiation, as most published studies have not specified how poor differentiation was defined. To avoid the risk of under-treatment in early CRC, the Royal College of Pathologists re-commend grading on the worst area in local excision specimens until further data are available[19]. In contrast, a major resection specimen is graded based on the predominant area of the tumour.

Whilst poor differentiation is generally accepted to be a poor prognostic feature, an exception in which it is associated with a more favourable stage-adjusted prognosis is in patients with dMMR, seen in 12%-15% of all cases[23,24]. Most dMMR cases are due to somatic epigenetic silencing of the MLH1 gene but a minority of cases are due to Lynch syndrome. From the current literature it is unclear if patients with poorly differentiated pT1 dMMR CRCs have a lower risk of LNM than poorly differentiated pT1 CRCs without dMMR. However, given the rarity of metastatic disease in dMMR CRC it is likely that poor differentiation is only an adverse factor in cases with proficient mismatch repair. Further evidence is required to confirm this hypothesis. Mismatch repair immunohistochemistry or alternative technologies including microsatellite instability testing should therefore be considered mandatory when determining LNM risk in local excision specimens.

Venous, lymphatic and perineural invasion

The presence of submucosal lymphatic invasion[7,17,25,26] [relative risk (RR) = 5.2, 95% confidence interval (CI): 4.0-6.8[7]] and to a much lesser extent venous invasion (RR = 2.2; 95%CI: 1.4 -3.2[7]) and perineural invasion[27] have been shown to be some of the

strongest predictors of LNM in early CRC. It is therefore important to carefully assess for their presence and report these factors separately rather than stating the presence "lymphovascular invasion" for example. The location of the deepest point of involvement (either intramural or extramural) should be specified as this is also prognostic[28]. In the context of a local excision, the deepest point visible will usually be intramural. Lymphatic invasion is well recognised to be subjective with significant rates of inter-observer variation[29]. This can be caused by difficulties distinguishing lymphatics from venules, retraction artefacts, tumour budding and poorly differentiated clusters[29,30]. In cases of doubt, D2-40 immunohistochemistry can be helpful to confirm the presence of a lymphatic channel and elastin stains can be helpful to identify veins[25]. The use of such ancillary stains has been shown to significantly improve the inter-observer agreement[31].

Resection margin status

In polypectomy/local excision specimens, the status of the resection margin in conjunction with other high risk histopathology factors determines the risk of local recurrence. Tumours which are present at the resection margin or within the diathermised zone should be considered for further treatment regardless of high risk factors. In cases where the invasive tumour extends to the peripheral resection margin only, a repeat endoscopy and further local excision should be considered[19].

There is currently significant controversy about the degree of risk in cases where a tumour extends close to the deep resection margin (1 mm or less) but does not directly involve it. Within the NHS BCSP, the recently revised pathological reporting guidance has maintained 1 mm as the optimal cut-off to define margin involvement in order to reduce the risk of incomplete resection, despite the risk that this strategy will lead to a higher rate of major bowel surgery. Despite this guidance, in the absence of any other high-risk histopathology features, local re-excision may be a reasonable treatment option[11,32,33].

Level of submucosal invasion

Depending on the shape of the lesion (pedunculated or non-pedunculated), and the status of the muscularis mucosa (identifiable or non-identifiable), different classification systems may be used to define the level of submucosal invasion. A qualitative assessment of sessile lesions was initially proposed by Kudo *et al*[34] which separated the submucosa into thirds: sm1 (superficial); sm2 (middle); and sm3 (deep). Invasion into sm3 has been associated with a higher risk of LNM when compared to invasion confined to sm1/sm2 (RR = 3.6, 95%CI: 1.3-9.8)[7]. This method was further subsequently modified into a semi-quantitative system by Kikuchi (sm1: Invasion up to 0.2-0.3 mm; sm2: Intermediate invasion; sm3: Invasion near the muscularis propria)[35]. A third quantitative measurement with a clear cut-off defining the levels as sm1: Up to 0.5mm; sm2: 0.5-1.0 mm; sm3: Beyond 1.0 mm[36]. A second classification system was proposed by Haggitt for pT1 CRC with a polypoid shape, which assesses the depth of invasion into four levels, with level four invasion described as an adverse factor[37].

Several issues arise when attempting to apply these two classification systems in a routine clinical setting. Firstly, the received sample can become fragmented or suboptimally orientated on histological sections meaning that accurate assessment is not possible. To apply the Kudo system, the muscularis propria needs to be visible which is not usually present in local excisions (with the exception of full thickness transanal resection specimens). Without the muscularis propria indicating that the full thickness of the submucosa is included, accurate division of the submucosa into thirds is impossible. Haggitt classification can also be difficult to apply in poorly orientated specimens or polyps that are lacking a clear stalk. These limitations and difficulties show the need for better alternative measures that reply on quantitative parameters.

NOVEL HISTOPATHOLOGICAL PARAMETERS

Depth of submucosal invasion

Studies, in particular of Japanese populations, have identified the absolute depth of submucosal invasion as an important quantitative factor for predicting lymph node metastasis[38,39]. Ueno *et al*[11] proposed that the absolute depth of invasion beyond the muscularis mucosa and the width of the invasive tumour are more objective parameters than the conventional factors described above[11,17]. On univariate analysis, submucosal invasion ≥ 1 mm was predictive for LNM in pT1 CRC (RR = 5.2, 95%CI: 1.8-15.4)[7].

The current guidelines from the Japanese Society for Cancer of the Colon and

Rectum recommend major resection if the cancer has an involved deep margin or if one of the following high risk factors is present: poor differentiation, signet-ring cell carcinoma, mucinous carcinoma, depth of invasion > 1000 μm, vascular invasion or budding G2/G3[14]. Interestingly a study in the Japanese population found that submucosal invasion depth > 1000 μm alone would lead to approximately 80% of malignant polyps being treated with laparotomy[39]. This approach has not been routinely adopted in western populations as it would cause a significant increase in the major resection rate in patients with a significantly higher risk of post-operative mortality. In addition, there is only limited data on the prediction of LNM according to the absolute depth of submucosal invasion in western populations[40].

Area of submucosal invasion

Three dimensional histological reconstructions of the large intestinal submucosa has demonstrated that the number and size of blood and lymphatic vessels does not increase towards the base of the submucosa as expected[41,42]. There are a greater number of vessels in sm1 and the vessels are largest in sm2, suggesting that the absolute depth of submucosal invasion may not be the most important parameter. Pilot studies in a western populations have suggested that the width of the cancer (cut-off 11.5 mm; $P = 0.001$) and the area of submucosal invasion (cut-off 35 mm^2; $P < 0.001$) were significantly associated with the risk of LNM and showed superior prediction when compared to the depth of invasion[40]. Taken together, it is highly likely that the absolute area of submucosal invasion in sm1/sm2 where the majority of vessels are located is the best predictor of LNM. With the routine adoption of digital pathology, measuring the area of submucosal invasion is now readily feasible (Figure 1).

Tumour budding

The predictive value of tumour budding for LNM has been demonstrated for various cancers[43-46], but implementation into routine guidelines has been hindered by a lack of practical guidance on assessment. Multiple different systems are described in the literature leading to confusion over which system should be used[47]. Recently an international consensus group agreed the definition of budding as "a single cancer cell or a cell cluster of up to four tumour cells"[48] as well as detailing the practical steps that should be taken to evaluate this marker (Figure 2). This led to the implementation of tumour budding as an additional prognostic factor in the eighth edition of the Union for International Cancer Control's (UICC's) TNM classification. Tumour budding has been included in the College of American Pathologists (CAP) guidelines but not yet as a core factor in the UK guidelines until sufficient evidence of reproducibility and its role in patient risk stratification exists[19]. Nevertheless some studies have shown that the inter-observer variation in tumour budding is improved by the international consensus definition, although further studies in western populations are needed given that most of the large studies to date have been performed in Asian populations.

Poorly differentiated clusters

A new emerging risk factor for LNM in pT1 CRC is the presence of poorly differentiated clusters (PDC), which are defined as "malignant clusters with five or more cells lacking glandular differentiation"[49,50] (Figure 2). Studies have shown that the presence of PDC is a strong predictor of LNM with greater reproducibility than tumour differentiation or budding in a study of 3,556 pT1 CRC [OR = 3.3 (95%CI: 2.6-4.1) $P < 0.0001$]. Further validation work is now required to confirm the importance of PDC in early CRC and define the optimal risk cut-offs in addition to clear practical guidance on the method of assessment[49].

Proportion of stroma

An emerging quantitative prognostic factor is the proportion of stroma within the overall tumour area[51]. It has been shown in a number of different cancers, including CRC, that a greater proportion of stroma is a poor prognostic factor[52-55]. The proportion of stroma can be subjectively estimated by the tumour stroma ratio[56] or accurately quantitated by cell density measurements[54]. It is thought that in CRC the high stroma group correlates with CMS4 consensus molecular subtyping, with both groups accounting for around 25% of cases with the poorest prognosis[57-59]. The prediction of LNM in pT1 CRC according to the proportion of stroma is as yet unknown but studies are ongoing.

Tumor immunology

The quantitation of the total number of tumour infiltrating lymphocytes has been shown to correlate with prognosis in CRC and other cancer types[60-62]. A study of 29

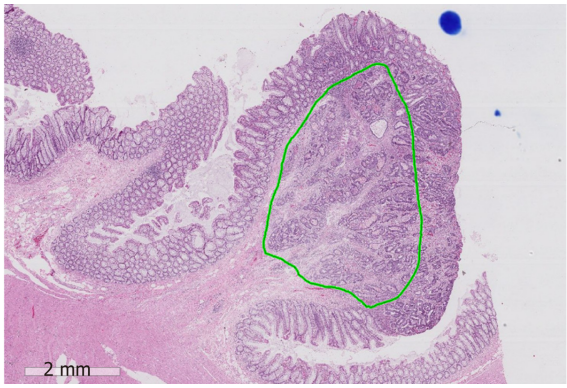


Figure 1 An example showing measurement of the area of submucosal invasion on a digital slide of a pT1 colorectal cancer (green area). The position of the destroyed muscularis mucosae has been estimated. The scale bar signifies 2 mm.

early stage CRC (stage I and II) showed that the combination of CD8(+) plus CD45RO(+) cells could be predictive for tumour recurrence and survival[63]. An increase in specific lymphocyte populations has been shown to strongly predict prognosis in CRC. The best-reported system in the recent literature is the Immunoscore®, which is generated on the basis of CD3 and CD8 expression in the tumour[64-66]. Automated algorithms to assess the number of lymphocytes are currently being explored. Again, the prediction of LNM in pT1 CRC according to tumour immunology is as yet unknown.

META-ANALYSIS OF HISTOPATHOLOGICAL PARAMETERS

A meta-analysis from 2011 included 17 studies with a total of 3621 patients and showed that the presence of lymphatic invasion (RR = 5.2, 95%CI: 4.0-6.8), high tumour budding (RR = 5.1, 95%CI: 3.6-7.3), submucosal invasion ≥ 1 mm (RR = 5.2, 95%CI: 1.8-15.4), and poor differentiation (RR = 4.8, 95%CI: 3.3-6.9) were associated with a higher risk of LNM in pT1 CRC on univariate analysis[7]. Another fixed-effects meta-analysis included 76 studies and showed that lymphatic invasion (OR = 8.62) was the strongest factor, followed by tumour depth (pT2 *vs* pT1; OR = 2.62) and tumour differentiation (OR = 2.38) in predicting LNM[26]. In a subset analysis, poor differentiation at the invasive front (OR = 6.08) and tumour budding (OR = 5.82) were the most predictive in rectal cancer[26]. A meta-analysis from 2013 included 23 studies with 4510 patients and demonstrated similar findings, with a greater risk of LNM in pT1 CRC with a depth of submucosal invasion of > 1 mm (OR = 3.87, 95%CI: 1.50-10.00, $P = 0.005$), lymphovascular invasion (OR = 4.81, 95%CI: 3.14-7.37, $P < 0.00001$), poor differentiation (OR = 5.60, 95%CI: 2.90-10.82, $P < 0.00001$) or tumour budding (OR = 7.74, 95%CI: 4.47-13.39, $P < 0.001$)[17]. Finally, a meta-analysis in early CRC included 41 studies with 10137 patients and showed a strong association between the presence of tumour budding and risk of LNM in pT1 CRC (OR = 6.44; 95%CI: 5.26-7.87; $P < 0.0001$)[67].

MULTIVARIATE RISK STRATIFICATION MODELS

The univariate analyses described above have identified a number of conventional and novel histopathological risk factors for LNM in pT1 CRC. To date, very few studies have proposed multivariate risk prediction models and currently there is no single risk stratification model used internationally. Ueno *et al*[11] demonstrated that the best risk multivariate prediction model in a cohort of 251 cases included poor differentiation (OR = 2.9; 95%CI: 1.2-7.4; $P = 0.023$), vascular invasion (OR = 2.7; 95%CI: 1.1-7.0; $P = 0.039$), tumour budding (OR = 3.7; 95%CI: 1.4-9.9; $P = 0.008$) and width of submucosal invasion (≥ 4000 μm)[11]. Another study of 140 cases used a logistic regression analysis and built an algorithm based on lymphatic invasion (OR = 1.45, $P < 0.05$), absence of lymphocyte infiltration (OR = 16.6, $P = 0.016$), cribriform-type structural atypia (OR = 3.7; $P < 0.05$), venous invasion (OR = 3.26, $P < 0.05$), and depth of invasion (cut-off > 2 mm; OR = 1.45, $P < 0.05$)[68]. Lymphocyte infiltration was investigated in the invasive area of submucosal carcinoma and classified as either

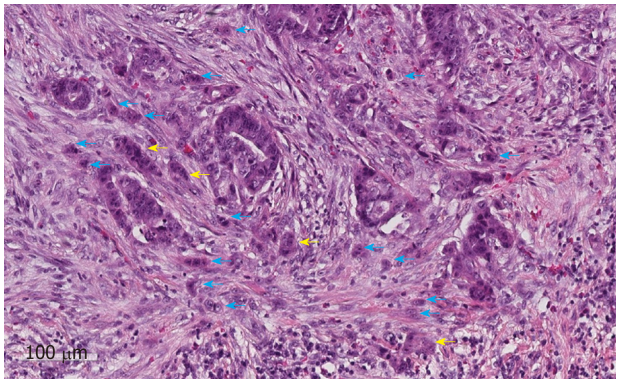


Figure 2 An example of tumour budding (blue arrows) and poorly differentiated clusters (yellow arrows) at the invasive edge of a pT1 colorectal cancer. The scale bar signifies 100 μm.

negative (no or little infiltration) or positive (follicular structures or infiltration by more lymphocytes than the number of tumour cells).

A larger retrospective study of 806 cases showed that independent predictors of LNM in multivariate analysis were: depth of submucosal invasion ≥ 1000 μm (OR = 5.56; 95%CI: 2.14-19.10) and high-grade budding (OR = 3.14; 95%CI: 1.91-5.21). High-grade budding was defined as five or more buds per high power field (0.95 mm^2). The study proposed a three-tier risk classification system based on the depth of submucosal invasion and budding: high-risk with a depth of submucosal invasion ≥ 1000 μm and high-grade budding, intermediate-risk with a depth of submucosal invasion ≥ 1000 μm and low-grade budding, and low-risk with a depth of submucosal invasion < 1000 μm. Additional factors for used to subclassify the intermediate risk group further were lymphovascular invasion and differentiation grade[69].

A recently published study proposed a risk stratification model for pedunculated pT1 CRC (3% to 7% LNM rate in Asian populations)[9,10,39,69] and investigated the following six factors: tumour differentiation; submucosal invasion depth by Haggitt classification; lymphovascular invasion; tumour budding; PDCs; and the condition of the muscularis mucosae[70]. Tumour budding and PDC were defined and graded as above but assessed in a 0.785 mm^2 field (grade 1: 0-4; Grade 2: 5-9; Grade 3: 10 or more). The status of the muscularis mucosa was classified as Type A: Shattered but aligned muscularis mucosa or Type B: Incompletely or completely disrupted muscularis mucosae. The authors ultimately recommended a new model including the following four risk factors: lymphovascular invasion; Haggitt level 4 invasion; muscularis mucosae type B (incompletely or completely disrupted); high grade PDCs/tumour budding (both grade 2-3). High-grade tumour budding and high-grade PDC were grouped together as "any kind of positive budding". The model had a sensitivity of 83.8% and specificity of 70.3% for predicting LNM (area under the curve value of 0.83), and would classify 32% of all cases in the high-risk group, 68% in the low risk group and miss 1.3% of all LNM.

Unfortunately the majority of these studies include retrospective series with small numbers of patients. Due to the 10%-15% rate of LNM in pT1 CRC, only a limited number of events are present leading to limited data in both Western and Asian populations. Well-designed prospective cohort studies are urgently needed to define robust international standards[49,69]. Ultimately a validated multivariate risk stratification model including both clinical and histopathological factors could be used to define prognosis in a similar way to that already developed for breast cancer.

DISCUSSION

In this review we have summarised the main conventional histopathological risk factors for LNM in pT1 CRC in addition to a number of novel factors that have recently been proposed. It is well recognised that parameters including lymphovascular invasion, poor differentiation, and depth of submucosal invasion are significantly correlated with an increased risk of LNM. Unfortunately, lymphovascular invasion and tumour differentiation grading show high levels of inter-observer variation, which limits their clinical usefulness. Univariate markers for LNM in pT1 CRC should therefore be interpreted with caution when deciding whether to proceed with major bowel resection after local excision due to the significant risks of major morbidity and mortality.

With the routine implementation of digital pathology into clinical diagnostics[20-22], novel histopathological markers including the absolute depth of invasion, area of submucosal invasion and proportion of stroma can now be easily measured. Due to the quantitative nature of these parameters, it is likely that they are considerably more reproducible than the traditional subjective parameters. However, their evaluation can take a considerable amount of time to perform manually, hence there is a clear role for artificial intelligence, which is also likely to improve the reproducibility of con-ventional and novel risk factors.

Multivariate risk stratification models need to be developed and validated to optimise the management of pT1 CRC. In the future, artificial intelligence on digital pathology slides could be applied to compare different multivariate risk stratification models to identify the optimal way to accurately predict LNM[71]. This will should reduce the inaccuracy associated with relying on individual subjective markers and has the potential to further refine the group at highest risk of LNM and therefore reduce the overall number of patients exposed to the risks of major bowel resection. By combining the histopathological data with molecular data, patient data and treatment data, a personalised risk stratification model could be created with the aim of determining the optimal treatment pathway for individual patients[49].

CONCLUSION

In this review we present the various histopathological risk factors for predicting systemic spread in pT1 colorectal cancer and introduce potential novel quantitative variables and multivariable risk models that could be used to better define the optimal treatment of this increasingly common disease.

REFERENCES

1 **Siegel RL**, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin* 2018; **68**: 7-30 [PMID: 29313949 DOI: 10.3322/caac.21442]

2 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

3 **Amri R**, Bordeianou LG, Sylla P, Berger DL. Impact of screening colonoscopy on outcomes in colon cancer surgery. *JAMA Surg* 2013; **148**: 747-754 [PMID: 23784448 DOI: 10.1001/jamasurg.2013.8]

4 **Morris EJ**, Penegar S, Whitehouse LE, Quirke P, Finan P, Bishop DT, Wilkinson J, Houlston RS. A retrospective observational study of the relationship between family history and survival from colorectal cancer. *Br J Cancer* 2013; **108**: 1502-1507 [PMID: 23511565 DOI: 10.1038/bjc.2013.91]

5 **The International Agency for Research on Cancer**. WHO Classification of Tumours of the Digestive System. Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. World health organization international histological classification of tumours, 4th ed. 2010; 3

6 **Ikematsu H**, Yoda Y, Matsuda T, Yamaguchi Y, Hotta K, Kobayashi N, Fujii T, Oono Y, Sakamoto T, Nakajima T, Takao M, Shinohara T, Murakami Y, Fujimori T, Kaneko K, Saito Y. Long-term outcomes after resection for submucosal invasive colorectal cancers. *Gastroenterology* 2013; **144**: 551-559; quiz e14 [PMID: 23232297 DOI: 10.1053/j.gastro.2012.12.003]

7 **Bosch SL**, Teerenstra S, de Wilt JH, Cunningham C, Nagtegaal ID. Predicting lymph node metastasis in pT1 colorectal cancer: a systematic review of risk factors providing rationale for therapy decisions. *Endoscopy* 2013; **45**: 827-834 [PMID: 23884793 DOI: 10.1055/s-0033-1344238]

8 **Hassan C**, Zullo A, Risio M, Rossini FP, Morini S. Histologic risk factors and clinical outcome in colorectal malignant polyp: a pooled-data analysis. *Dis Colon Rectum* 2005; **48**: 1588-1596 [PMID: 15937622 DOI: 10.1007/s10350-005-0063-3]

9 **Matsuda T**, Fukuzawa M, Uraoka T, Nishi M, Yamaguchi Y, Kobayashi N, Ikematsu H, Saito Y, Nakajima T, Fujii T, Murakami Y, Shimoda T, Kushima R, Fujimori T. Risk of lymph node metastasis in patients with pedunculated type early invasive colorectal cancer: a retrospective multicenter study. *Cancer Sci* 2011; **102**: 1693-1697 [PMID: 21627735 DOI: 10.1111/j.1349-7006.2011.01997.x]

10 **Asayama N**, Oka S, Tanaka S, Nagata S, Furudoi A, Kuwai T, Onogawa S, Tamura T, Kanao H, Hiraga Y, Okanobu H, Kuwabara T, Kunihiro M, Mukai S, Goto E, Shimamoto F, Chayama K. Long-term outcomes after treatment for pedunculated-type T1 colorectal carcinoma: a multicenter retrospective cohort study. *J Gastroenterol* 2016; **51**: 702-710 [PMID: 26573300 DOI: 10.1007/s00535-015-1144-2]

11 **Ueno H**, Mochizuki H, Hashiguchi Y, Shimazaki H, Aida S, Hase K, Matsukuma S, Kanai T, Kurihara H, Ozawa K, Yoshimura K, Bekku S. Risk factors for an adverse outcome in early invasive colorectal carcinoma. *Gastroenterology* 2004; **127**: 385-394 [PMID: 15300569]

12 **Marks KM**, West NP, Morris E, Quirke P. Clinicopathological, genomic and immunological factors in colorectal cancer prognosis. *Br J Surg* 2018; **105**: e99-e109 [PMID: 29341159 DOI: 10.1002/bjs.10756]

13 **Xu F**, Xu J, Lou Z, Di M, Wang F, Hu H, Lai M. Micropapillary component in colorectal carcinoma is associated with lymph node metastasis in T1 and T2 Stages and decreased survival time in TNM stages I and II. *Am J Surg Pathol* 2009; **33**: 1287-1292 [PMID: 19471152 DOI: 10.1097/PAS.0b013e3181a5387b]

14 **Watanabe T**, Muro K, Ajioka Y, Hashiguchi Y, Ito Y, Saito Y, Hamaguchi T, Ishida H, Ishiguro M, Ishihara S, Kanemitsu Y, Kawano H, Kinugasa Y, Kokudo N, Murofushi K, Nakajima T, Oka S, Sakai Y, Tsuji A, Uehara K, Ueno H, Yamazaki K, Yoshida M, Yoshino T, Boku N, Fujimori T, Itabashi M, Koinuma N, Morita T, Nishimura G, Sakata Y, Shimada Y, Takahashi K, Tanaka S, Tsuruta O, Yamaguchi T, Yamaguchi N, Tanaka T, Kotake K, Sugihara K; Japanese Society for Cancer of the Colon

and Rectum. Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2016 for the treatment of colorectal cancer. *Int J Clin Oncol* 2018; **23**: 1-34 [PMID: 28349281 DOI: 10.1007/s10147-017-1101-6]

15 **Halvorsen TB**, Seim E. Degree of differentiation in colorectal adenocarcinomas: a multivariate analysis of the influence on survival. *J Clin Pathol* 1988; **41**: 532-537 [PMID: 3384983]

16 **Compton CC**. Colorectal carcinoma: diagnostic, prognostic, and molecular features. *Mod Pathol* 2003; **16**: 376-388 [PMID: 12692203 DOI: 10.1097/01.MP.0000062859.46942.93]

17 **Beaton C**, Twine CP, Williams GL, Radcliffe AG. Systematic review and meta-analysis of histopathological factors influencing the risk of lymph node metastasis in early colorectal cancer. *Colorectal Dis* 2013; **15**: 788-797 [PMID: 23331927 DOI: 10.1111/codi.12129]

18 **Tateishi Y**, Nakanishi Y, Taniguchi H, Shimoda T, Umemura S. Pathological prognostic factors predicting lymph node metastasis in submucosal invasive (T1) colorectal carcinoma. *Mod Pathol* 2010; **23**: 1068-1072 [PMID: 20473277 DOI: 10.1038/modpathol.2010.88]

19 Dataset for histopathological reporting of colorectal cancer. September 2018 G049.

20 **Williams BJ**, Bottoms D, Treanor D. Future-proofing pathology: the case for clinical adoption of digital pathology. *J Clin Pathol* 2017; **70**: 1010-1018 [PMID: 28780514 DOI: 10.1136/jclinpath-2017-204644]

21 **Williams BJ**, Bottoms D, Clark D, Treanor D. Future-proofing pathology part 2: building a business case for digital pathology. *J Clin Pathol* 2019; **72**: 198-205 [PMID: 29549217 DOI: 10.1136/jclinpath-2017-204926]

22 **Williams BJ**, Hanby A, Millican-Slater R, Nijhawan A, Verghese E, Treanor D. Digital pathology for the primary diagnosis of breast histopathological specimens: an innovative validation and concordance study on digital pathology validation and training. *Histopathology* 2018; **72**: 662-671 [PMID: 28940580 DOI: 10.1111/his.13403]

23 **Kawakami H**, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. *Curr Treat Options Oncol* 2015; **16**: 30 [PMID: 26031544 DOI: 10.1007/s11864-015-0348-2]

24 **Ryan E**, Sheahan K, Creavin B, Mohan HM, Winter DC. The current value of determining the mismatch repair status of colorectal cancer: A rationale for routine testing. *Crit Rev Oncol Hematol* 2017; **116**: 38-57 [PMID: 28693799 DOI: 10.1016/j.critrevonc.2017.05.006]

25 **Wada H**, Shiozawa M, Katayama K, Okamoto N, Miyagi Y, Rino Y, Masuda M, Akaike M. Systematic review and meta-analysis of histopathological predictive factors for lymph node metastasis in T1 colorectal cancer. *J Gastroenterol* 2015; **50**: 727-734 [PMID: 25725617 DOI: 10.1007/s00535-015-1057-0]

26 **Glasgow SC**, Bleier JI, Burgart LJ, Finne CO, Lowry AC. Meta-analysis of histopathological features of primary colorectal cancers that predict lymph node metastases. *J Gastrointest Surg* 2012; **16**: 1019-1028 [PMID: 22258880 DOI: 10.1007/s11605-012-1827-4]

27 **Liebig C**, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, Berger DH, Albo D. Perineural invasion is an independent predictor of outcome in colorectal cancer. *J Clin Oncol* 2009; **27**: 5131-5137 [PMID: 19738119 DOI: 10.1200/JCO.2009.22.4949]

28 **Betge J**, Pollheimer MJ, Lindtner RA, Kornprat P, Schlemmer A, Rehak P, Vieth M, Hoefler G, Langner C. Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. *Cancer* 2012; **118**: 628-638 [PMID: 21751188 DOI: 10.1002/cncr.26310]

29 **Ishii M**, Ota M, Saito S, Kinugasa Y, Akamoto S, Ito I. Lymphatic vessel invasion detected by monoclonal antibody D2-40 as a predictor of lymph node metastasis in T1 colorectal cancer. *Int J Colorectal Dis* 2009; **24**: 1069-1074 [PMID: 19387662 DOI: 10.1007/s00384-009-0699-x]

30 **Wada H**, Shiozawa M, Sugano N, Morinaga S, Rino Y, Masuda M, Akaike M, Miyagi Y. Lymphatic invasion identified with D2-40 immunostaining as a risk factor of nodal metastasis in T1 colorectal cancer. *Int J Clin Oncol* 2013; **18**: 1025-1031 [PMID: 23114785 DOI: 10.1007/s10147-012-0490-9]

31 **Suzuki A**, Togashi K, Nokubi M, Koinuma K, Miyakura Y, Horie H, Lefor AT, Yasuda Y. Evaluation of venous invasion by Elastica van Gieson stain and tumor budding predicts local and distant metastases in patients with T1 stage colorectal cancer. *Am J Surg Pathol* 2009; **33**: 1601-1607 [PMID: 19574884 DOI: 10.1097/PAS.0b013e3181ae29d6]

32 **Brown IS**, Bettington ML, Bettington A, Miller G, Rosty C. Adverse histological features in malignant colorectal polyps: a contemporary series of 239 cases. *J Clin Pathol* 2016; **69**: 292-299 [PMID: 26424814 DOI: 10.1136/jclinpath-2015-203203]

33 **Gill MD**, Rutter MD, Holtham SJ. Management and short-term outcome of malignant colorectal polyps in the north of England(1). *Colorectal Dis* 2013; **15**: 169-176 [PMID: 22709241 DOI: 10.1111/j.1463-1318.2012.03130.x]

34 **Kudo S**. Endoscopic mucosal resection of flat and depressed types of early colorectal cancer. *Endoscopy* 1993; **25**: 455-461 [PMID: 8261988 DOI: 10.1055/s-2007-1010367]

35 **Kikuchi R**, Takano M, Takagi K, Fujimoto N, Nozaki R, Fujiyoshi T, Uchida Y. Management of early invasive colorectal cancer. Risk of recurrence and clinical guidelines. *Dis Colon Rectum* 1995; **38**: 1286-1295 [PMID: 7497841 DOI: 10.1007/Bf02049154]

36 **Yamamoto S**, Watanabe M, Hasegawa H, Baba H, Yoshinare K, Shiraishi J, Kitajima M. The risk of lymph node metastasis in T1 colorectal carcinoma. *Hepatogastroenterology* 2004; **51**: 998-1000 [PMID: 15239233]

37 **Haggitt RC**, Glotzbach RE, Soffer EE, Wruble LD. Prognostic factors in colorectal carcinomas arising in adenomas: implications for lesions removed by endoscopic polypectomy. *Gastroenterology* 1985; **89**: 328-336 [PMID: 4007423]

38 **Kawachi H**, Eishi Y, Ueno H, Nemoto T, Fujimori T, Iwashita A, Ajioka Y, Ochiai A, Ishiguro S, Shimoda T, Mochizuki H, Kato Y, Watanabe H, Koike M, Sugihara K. A three-tier classification system based on the depth of submucosal invasion and budding/sprouting can improve the treatment strategy for T1 colorectal cancer: a retrospective multicenter study. *Mod Pathol* 2015; **28**: 872-879 [PMID: 25720321 DOI: 10.1038/modpathol.2015.36]

39 **Kitajima K**, Fujimori T, Fujii S, Takeda J, Ohkura Y, Kawamata H, Kumamoto T, Ishiguro S, Kato Y, Shimoda T, Iwashita A, Ajioka Y, Watanabe H, Watanabe T, Muto T, Nagasako K. Correlations between lymph node metastasis and depth of submucosal invasion in submucosal invasive colorectal carcinoma: a Japanese collaborative study. *J Gastroenterol* 2004; **39**: 534-543 [PMID: 15235870 DOI: 10.1007/s00535-004-1339-4]

40 **Toh EW**, Brown P, Morris E, Botterill I, Quirke P. Area of submucosal invasion and width of invasion predicts lymph node metastasis in pT1 colorectal cancers. *Dis Colon Rectum* 2015; **58**: 393-400 [PMID: 25751795 DOI: 10.1097/DCR.0000000000000315]

41 **Roberts N**, Magee D, Song Y, Brabazon K, Shires M, Crellin D, Orsi NM, Quirke R, Quirke P, Treanor D. Toward routine use of 3D histopathology as a research tool. *Am J Pathol* 2012; **180**: 1835-1842 [PMID: 22490922 DOI: 10.1016/j.ajpath.2012.01.033]

42 **Brown PJ**, Toh EW, Smith KJ, Jones P, Treanor D, Magee D, Burke D, Quirke P. New insights into the lymphovascular microanatomy of the colon and the risk of metastases in pT1 colorectal cancer obtained with quantitative methods and three-dimensional digital reconstruction. *Histopathology* 2015; **67**: 167-175 [PMID: 25557923 DOI: 10.1111/his.12639]

43 **Almangush A**, Bello IO, Keski-Säntti H, Mäkinen LK, Kauppila JH, Pukkila M, Hagström J, Laranne J, Tommola S, Nieminen O, Soini Y, Kosma VM, Koivunen P, Grénman R, Leivo I, Salo T. Depth of invasion, tumor budding, and worst pattern of invasion: prognostic indicators in early-stage oral tongue cancer. *Head Neck* 2014; **36**: 811-818 [PMID: 23696499 DOI: 10.1002/hed.23380]

44 **Almangush A**, Salo T, Hagström J, Leivo I. Tumour budding in head and neck squamous cell carcinoma - a systematic review. *Histopathology* 2014; **65**: 587-594 [PMID: 24897954 DOI: 10.1111/his.12471]

45 **Masuda R**, Kijima H, Imamura N, Aruga N, Nakamura Y, Masuda D, Takeichi H, Kato N, Nakagawa T, Tanaka M, Inokuchi S, Iwazaki M. Tumor budding is a significant indicator of a poor prognosis in lung squamous cell carcinoma patients. *Mol Med Rep* 2012; **6**: 937-943 [PMID: 22940760 DOI: 10.3892/mmr.2012.1048]

46 **Jesinghaus M**, Strehl J, Boxberg M, Brühl F, Wenzel A, Konukiewitz B, Schlitter AM, Steiger K, Warth A, Schnelzer A, Kiechle M, Beckmann MW, Noske A, Hartmann A, Mehlhorn G, Koch MC, Weichert W. Introducing a novel highly prognostic grading scheme based on tumour budding and cell nest size for squamous cell carcinoma of the uterine cervix. *J Pathol Clin Res* 2018; **4**: 93-102 [PMID: 29665323 DOI: 10.1002/cjp2.95]

47 **Mitrovic B**, Schaeffer DF, Riddell RH, Kirsch R. Tumor budding in colorectal carcinoma: time to take notice. *Mod Pathol* 2012; **25**: 1315-1325 [PMID: 22790014 DOI: 10.1038/modpathol.2012.94]

48 **Lugli A**, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, El Zimaity H, Fléjou JF, Hansen TP, Hartmann A, Kakar S, Langner C, Nagtegaal I, Puppa G, Riddell R, Ristimäki A, Sheahan K, Smyrk T, Sugihara K, Terris B, Ueno H, Vieth M, Zlobec I, Quirke P. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. *Mod Pathol* 2017; **30**: 1299-1311 [PMID: 28548122 DOI: 10.1038/modpathol.2017.46]

49 **Ueno H**, Hase K, Hashiguchi Y, Shimazaki H, Yoshii S, Kudo SE, Tanaka M, Akagi Y, Suto T, Nagata S, Matsuda K, Komori K, Yoshimatsu K, Tomita Y, Yokoyama S, Shinto E, Nakamura T, Sugihara K. Novel risk factors for lymph node metastasis in early invasive colorectal cancer: a multi-institution pathology review. *J Gastroenterol* 2014; **49**: 1314-1323 [PMID: 24065123 DOI: 10.1007/s00535-013-0881-3]

50 **Ueno H**, Kajiwara Y, Shimazaki H, Shinto E, Hashiguchi Y, Nakanishi K, Maekawa K, Katsurada Y, Nakamura T, Mochizuki H, Yamamoto J, Hase K. New criteria for histologic grading of colorectal cancer. *Am J Surg Pathol* 2012; **36**: 193-201 [PMID: 22251938 DOI: 10.1097/PAS.0b013e318235edee]

51 **West RB**, van de Rijn M. Experimental approaches to the study of cancer-stroma interactions: recent findings suggest a pivotal role for stroma in carcinogenesis. *Lab Invest* 2007; **87**: 967-970 [PMID: 17700561 DOI: 10.1038/labinvest.3700666]

52 **Hutchins GGA**, Treanor D, Wright A, Handley K, Magill L, Tinkler-Hundal E, Southward K, Seymour M, Kerr D, Gray R, Quirke P; QUASAR trial collaborators and the UK National Cancer Research Institute Colorectal Cancer Clinical Studies Group. Intratumoral stromal morphometry predicts disease recurrence but not response to 5-fluorouracil-results from the QUASAR trial of colorectal cancer. *Histopathology* 2018; **72**: 391-404 [PMID: 28746977 DOI: 10.1111/his.13326]

53 **Wright AI**, Coe A, Dattani M, Toh E, Hutchins G, West N, Grabsch H, Magee D, Quirke P, Treanor D. Automatic Image Analysis to Calculate the Cancer: Stroma Ratio in Colorectal Cancer. *J Pathol* 2012; **228**: S41-S41

54 **West NP**, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, Treanor D, Quirke P, Grabsch H. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. *Br J Cancer* 2010; **102**: 1519-1523 [PMID: 20407439 DOI: 10.1038/sj.bjc.6605674]

55 **Tsujino T**, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N, Monden M. Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clin Cancer Res* 2007; **13**: 2082-2090 [PMID: 17404090 DOI: 10.1158/1078-0432.CCR-06-2191]

56 **van Pelt GW**, Sandberg TP, Morreau H, Gelderblom H, van Krieken JHJM, Tollenaar RAEM, Mesker WE. The tumour-stroma ratio in colon cancer: the biological role and its prognostic impact. *Histopathology* 2018; **73**: 197-206 [PMID: 29457843 DOI: 10.1111/his.13489]

57 **Guinney J**, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, De Sousa E Melo F, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Tabernero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP, Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; **21**: 1350-1356 [PMID: 26457759 DOI: 10.1038/nm.3967]

58 **Dienstmann R**, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer* 2017; **17**: 79-92 [PMID: 28050011 DOI: 10.1038/nrc.2016.126]

59 **Sveen A**, Bruun J, Eide PW, Eilertsen IA, Ramirez L, Murumägi A, Arjama M, Danielsen SA, Kryeziu K, Elez E, Tabernero J, Guinney J, Palmer HG, Nesbakken A, Kallioniemi O, Dienstmann R, Lothe RA. Colorectal Cancer Consensus Molecular Subtypes Translated to Preclinical Models Uncover Potentially Targetable Cancer Cell Dependencies. *Clin Cancer Res* 2018; **24**: 794-806 [PMID: 29242316 DOI: 10.1158/1078-0432.CCR-17-1234]

60 **Denkert C**, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, Budczies J, Huober J, Klauschen F, Furlanetto J, Schmitt WD, Blohmer JU, Karn T, Pfitzner BM, Kümmel S, Engels K, Schneeweiss A, Hartmann A, Noske A, Fasching PA, Jackisch C, van Mackelenbergh M, Sinn P, Schem C, Hanusch C, Untch M, Loibl S. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol* 2018; **19**: 40-50 [PMID: 29233559 DOI: 10.1016/S1470-2045(17)30904-X]

61 **Savas P**, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, Loi S. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. *Nat Rev Clin Oncol* 2016; **13**: 228-241 [PMID: 26667975 DOI: 10.1038/nrclinonc.2015.215]

62 **Jass JR**, Ajioka Y, Allen JP, Chan YF, Cohen RJ, Nixon JM, Radojkovic M, Restall AP, Stables SR, Zwi

LJ. Assessment of invasive growth pattern and lymphocytic infiltration in colorectal cancer. *Histopathology* 1996; **28**: 543-548 [PMID: 8803598]

63 **Pagès F**, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, Lagorce C, Wind P, Marliot F, Bruneval P, Zatloukal K, Trajanoski Z, Berger A, Fridman WH, Galon J. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009; **27**: 5944-5951 [PMID: 19858404 DOI: 10.1200/JCO.2008.19.6147]

64 **Galon J**, Pagès F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Mihm M, Vidal-Vanaclocha F, Allison JP, Gnjatic S, Hakansson L, Huber C, Singh-Jasuja H, Ottensmeier C, Zwierzina H, Laghi L, Grizzi F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O'Donnell-Tormey J, Lagorce C, Pawelec G, Nishimura MI, Hawkins R, Lapointe R, Lundqvist A, Khleif SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D'Arrigo C, Kopetz S, Sinicrope FA, Trinchieri G, Gajewski TF, Ascierto PA, Fox BA. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* 2012; **10**: 205 [PMID: 23034130 DOI: 10.1186/1479-5876-10-205]

65 **Mlecnik B**, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, Church SE, Lafontaine L, Fischer M, Fredriksen T, Sasso M, Bilocq AM, Kirilovsky A, Obenauf AC, Hamieh M, Berger A, Bruneval P, Tuech JJ, Sabourin JC, Le Pessot F, Mauillon J, Rafii A, Laurent-Puig P, Speicher MR, Trajanoski Z, Michel P, Sesboüe R, Frebourg T, Pagès F, Valge-Archer V, Latouche JB, Galon J. Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* 2016; **44**: 698-711 [PMID: 26982367 DOI: 10.1016/j.immuni.2016.02.025]

66 **Pagès F**, Mlecnik B, Marliot F, Bindea G, Ou FS, Bifulco C, Lugli A, Zlobec I, Rau TT, Berger MD, Nagtegaal ID, Vink-Börger E, Hartmann A, Geppert C, Kolwelter J, Merkel S, Grützmann R, Van den Eynde M, Jouret-Mourin A, Kartheuser A, Léonard D, Remue C, Wang JY, Bavi P, Roehrl MHA, Ohashi PS, Nguyen LT, Han S, MacGregor HL, Hafezi-Bakhtiari S, Wouters BG, Masucci GV, Andersson EK, Zavadova E, Vocka M, Spacek J, Petruzelka L, Konopasek B, Dundr P, Skalova H, Nemejcova K, Botti G, Tatangelo F, Delrio P, Ciliberto G, Maio M, Laghi L, Grizzi F, Fredriksen T, Buttard B, Angelova M, Vasaturo A, Maby P, Church SE, Angell HK, Lafontaine L, Bruni D, El Sissy C, Haicheur N, Kirilovsky A, Berger A, Lagorce C, Meyers JP, Paustian C, Feng Z, Ballesteros-Merino C, Dijkstra J, van de Water C, van Lent-van Vliet S, Knijn N, Mușină AM, Scripcariu DV, Popivanova B, Xu M, Fujita T, Hazama S, Suzuki N, Nagano H, Okuno K, Torigoe T, Sato N, Furuhata T, Takemasa I, Itoh K, Patel PS, Vora HH, Shah B, Patel JB, Rajvik KN, Pandya SJ, Shukla SN, Wang Y, Zhang G, Kawakami Y, Marincola FM, Ascierto PA, Sargent DJ, Fox BA, Galon J. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* 2018; **391**: 2128-2139 [PMID: 29754777 DOI: 10.1016/S0140-6736(18)30789-X]

67 **Cappellesso R**, Luchini C, Veronese N, Lo Mele M, Rosa-Rizzotto E, Guido E, De Lazzari F, Pilati P, Farinati F, Realdon S, Solmi M, Fassan M, Rugge M. Tumor budding as a risk factor for nodal metastasis in pT1 colorectal cancers: a meta-analysis. *Hum Pathol* 2017; **65**: 62-70 [PMID: 28438617 DOI: 10.1016/j.humpath.2017.04.013]

68 **Egashira Y**, Yoshida T, Hirata I, Hamamoto N, Akutagawa H, Takeshita A, Noda N, Kurisu Y, Shibayama Y. Analysis of pathological risk factors for lymph node metastasis of submucosal invasive colon cancer. *Mod Pathol* 2004; **17**: 503-511 [PMID: 15001992 DOI: 10.1038/modpathol.3800030]

69 **Kobayashi H**, Higuchi T, Uetake H, Iida S, Ishikawa T, Ishiguro M, Sugihara K. Resection with en bloc removal of regional lymph node after endoscopic resection for T1 colorectal cancer. *Ann Surg Oncol* 2012; **19**: 4161-4167 [PMID: 22772868 DOI: 10.1245/s10434-012-2471-7]

70 **Backes Y**, Elias SG, Groen JN, Schwartz MP, Wolfhagen FHJ, Geesing JMJ, Ter Borg F, van Bergeijk J, Spanier BWM, de Vos Tot Nederveen Cappel WH, Kessels K, Seldenrijk CA, Raicu MG, Drillenburg P, Milne AN, Kerkhof M, Seerden TCJ, Siersema PD, Vleggaar FP, Offerhaus GJA, Lacle MM, Moons LMG; Dutch T1 CRC Working Group. Histologic Factors Associated With Need for Surgery in Patients With Pedunculated T1 Colorectal Carcinomas. *Gastroenterology* 2018; **154**: 1647-1659 [PMID: 29366842 DOI: 10.1053/j.gastro.2018.01.023]

71 **Ichimasa K**, Kudo S, Mori Y, Misawa M, Fumio I. Artificial Intelligence Can Accurately Predict the Presence of Lymph Node Metastasis in Pt1 Colorectal Cancers. *Gastrointestinal Endoscopy* 2017; **85**: Ab377-Ab377

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2898-2910

DOI: 10.3748/wjg.v25.i23.2898

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

NIMA related kinase 2 promotes gastric cancer cell proliferation *via* ERK/MAPK signaling

Wei-Dong Fan, Tao Chen, Peng-Jun Liu

ORCID number: Wei-Dong Fan (0000-0002-8545-150X); Tao Chen (0000-0001-5276-3913); Peng-Jun Liu (0000-0002-6700-9072).

Author contributions: Fan WD and Liu PJ designed the research; Fan WD and Chen T performed the research; Fan WD and Chen T analyzed the data; Liu PJ wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of the Wujin Hospital Affiliated to Jiangsu University.

Conflict-of-interest statement: The authors declare no financial and personal relationships with other people or organizations.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Wei-Dong Fan, Department of Gastroenterology, the Affiliated Zhangjiagang Hospital of Soochow University, Zhangjiagang 215600, Jiangsu Province, China

Tao Chen, Peng-Jun Liu, Department of Gastroenterology, the Wujin Hospital Affiliated to Jiangsu University, Changzhou 213002, Jiangsu Province, China

Corresponding author: Peng-Jun Liu, MD, Attending Doctor, Chief Doctor, Department of Gastroenterology, the Wujin Hospital Affiliated to Jiangsu University, No. 2, Yongning North Road, Changzhou 213002, Jiangsu Province, China. lpjlx2008@126.com
Telephone: +86-519-5336190
Fax: +86-519-5336190

Abstract

BACKGROUND

NIMA related kinase 2 (NEK2) is closely related to mitosis, and it is currently considered to be over-expressed frequently in many poorly prognostic cancers. However, the effect of the up-regulated NEK2 on cellular signaling in tumors, such as gastric cancer (GC), is con-fusing.

AIM

To determine the role of the up-regulation of NEK2 in GC.

METHODS

To investigate the pathological significance of NEK2 in GC, the expression pattern of NEK2 in GC was investigated based on the "Oncomain" database and compared between 30 pairs of cancer samples and adjacent tissues. The co-expression of NEK2 and ERK in GC was analyzed using The Cancer Genome Atlas (TCGA) database and confirmed in clinical samples by quantitative real-time PCR (qRT-PCR), and the survival curve was also plotted. Western blot or qRT-PCR was used to analyze the effect of NEK2 on the phosphorylation levels of ERK and c-JUN in two GC cell lines (BGC823 and SGC7901) with NEK2 overexpression, and the expression of the downstream effector cyclin D1. Furthermore, CCK8, EdU incorporation assay, and flow cytometry were used to detect the proliferative ability of BGC823 and SGC7901 cells with stably silenced ERK.

RESULTS

NEK2 was significantly up-regulated in human GC tissues. ERK was significantly associated with NEK2 expression in human clinical specimens, and combined overexpression of NEK2 and ERK potentially forecasted a poor prognosis and

Received: April 4, 2019
Peer-review started: April 4, 2019
First decision: April 11, 2019
Revised: April 27, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019
Published online: June 21, 2019

P-Reviewer: Jones G, Tanabe S
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

survival in GC patients. NEK2 knockdown in GC cells inhibited ERK and c-JUN phosphory-lation and reduced the transcription of cyclin D1. More interestingly, NEK2 can rescue the inhibition of cellular viability, proliferation, and cell cycle progression due to ERK knockdown.

CONCLUSION
Our results indicate that NEK2 plays a carcinogenic role in the malignant proliferation of GC cells *via* the ERK/MAPK signaling, which may be important for treatment and improving patient survival.

Key words: NIMA related kinase 2; ERK/MAPK signaling; Cyclin D1; Cell proliferation; Gastric cancer

Core tip: Expression of NIMA related kinase 2 (NEK2) is significantly up-regulated in human gastric cancer (GC) tissues and cells. Combined NEK2 and ERK expression may predict overall survival in patients with GC. NEK2 regulates the expression of cyclin D1 by affecting the phosphorylation of ERK and c-JUN in GC and mediates cell proliferation *via* the ERK/c-JUN/cyclin D1 axis.

Citation: Fan WD, Chen T, Liu PJ. NIMA related kinase 2 promotes gastric cancer cell proliferation *via* ERK/MAPK signaling. *World J Gastroenterol* 2019; 25(23): 2898-2910
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2898.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2898

INTRODUCTION

Gastric cancer (GC) is a type of malignant tumor in the digestive system with high incidence, and it is the third cause of human cancer-related death[1-3]. Although surgery, radiation therapy, and chemotherapy can improve patient survival to some extent, the 5-year survival rate of GC patients is still low (25%-30%), partly because the complexity of clinical heterogeneity[4-6]. Due to high cell proliferation is associated with poor survival in patients with GC[7], exploring the mechanisms of malignant proliferation of GC cells is important for the treatment and improvement of patient survival[8].

In mammals, NIMA related kinase 2 (NEK2), as an S/G2 phase kinase, usually shows a high expression level in various forms of cancer, mediating the cellular mitosis during cell division[9-11]. *In vivo*, silencing NEK2 can significantly slow tumor growth in mice and result in reduced tumor burden in tumor-forming organs[12-14]. Furthermore, some studies have shown that up-regulated NEK2 leads to malignant proliferation and drug resistance *in vivo*, and NEK2 siRNA can significantly reduce tumor formation ability in allogeneic tumor formation studies[15,16]. Although the exact molecular mechanism of NEK2 in the regulation of tumor formation and progression is largely unclear, the fact that NEK2 silencing induces the slower proliferation of cells suggests that NEK2 inhibition can resist tumor progression *in vivo*, and inhibition of NEK2 expression may become an effective treatment for GC[14,17-20].

The ERK/MAPK pathway communicates intracellular phosphorylation kinase signaling from a receptor on the surface of the cell to the DNA in the nucleus, and produces some changes in cell processes to respond to the extracellular stress[21,22]. Overall, once the extracellular mitogen binds to the receptor on the membrane, ERK will be activated and its downstream effector c-Jun will also be switched by transferring a phosphate group[23], participating in the ERK/MAPK signal transduction[24,25]. Many studies show that activation of the ERK/MAPK pathway plays an important role in tumorigenesis, progression, survival in GC[26-28].

Here, we showed that NEK2 promotes gastric tumorigenesis by regulating the cell cycle. Based on the rise of NEK2 expression in GC and the application of bioinformatics and clinical sample analysis, our study focused on the combined expression of NEK2 and ERK in GC and demonstrated that up-regulation of NEK2 in GC cells by targeting ERK/c-JUN/cyclin D1 signaling effectively promoted the proliferation of GC cells and significantly affected the clinical prognosis of GC

patients. Our study revealed the combined role of the NEK2 and ERK/MAPK pathways in the cell cycle, which is important for the GC treatment and improvement of patient survival.

MATERIALS AND METHODS

Human tissue specimens and cell lines

GC and paired adjacent tissues were obtained from 30 patients treated at Zhang jiagang First People's Hospital from 2016 to 2018. All patients had no history of chemotherapy or immunotherapy, and all samples were classified as GC by two pathologists. The sample use was approved by the patients and the Hospital Ethics Committee, and all patients provided informed consent.

The AGS, MGC803, BGC823, and SGC7901 cell lines and human normal gastric epithelial cell line (GES1) used in the study were purchased from the Chinese Academy of Medical Sciences Cell Resource Platform (Beijing, China). All cell lines were cultured in DMEM (Gibco) containing 10% FBS (Gibco) and 1% antibiotic (Gibco).

Cell transfection and lentivirus transductions

We transfected siRNA oligos into cells using Lipofectamine 3000 (Invitrogen). SiRNAs against NEK2 (siNEK2-1, 5'- GUCAGAUAUUGAGAAAAAUUACCAA-3'; siNEK2-2, 5'- AAUGAGAAAUCAGAUAUCUGGUCAT-3') and control siRNA [siN.C, 5'-UUCUCCGAACGUGUCACGUd(TT)-3'] were obtained from GenePharma (Suzhou). After 48 h of transfection, cells were harvested for subsequent experiments.

The shRNA targeting the ERK gene (5'- AACCTTTAAAGACTGATATTCAAAT-3') was designed and cloned into the pGV307-RFP vector (Addgene). Lentiviral packaging, purification, and infection of the cells of interest were performed according to the protocol provided by the manufacturer. Cells with stable ERK knockdown were selected using 5 μg/mL puromycin (Sigma) at 3 d after infection.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted from cells or tissue samples using TRIzol Reagent (Sigma), and 2 μg of RNA was transcribed into cDNA with a Prim primer RT Master Mix Kit (Takara) using random primers, followed by qRT-PCR using GoTaq qRT-PCR Master Mix (Promega, cat). The ΔΔCT method was used to calculate the expression level of the target mRNA. Experiments were performed in triplicate. The primers used in our study are listed in Table 1.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized and dehydrated, and immunohistochemical staining for NEK2 and ERK in tissues was performed using a DAB substrate kit (Maxin). The staining intensity and staining range were respectively scored using the standard method, and according to the final score (staining intensity × staining range), a score of less than 6 was considered to be low staining, and > 6 high staining.

Western blot analysis

Total cellular protein was extracted using RIPA lysis containing protease and phosphatase inhibitors. Protein concentration was measured according to the instructions of the BCA kit (Thermo Scientific). SDS-PAGE separation and immunoblotting were then performed according to standard methods. The primary antibodies used in our study are listed below: EK2 (Abcam), ERK1/2 (Cell Signaling Technology), c-JUN (Cell Signaling Technology), phospho-Erk1/2 (Thr202/Tyr204) (Cell Signaling Technology), phosphorylated c-JUN (Ser63) (Cell Signaling Technology), cyclin D1 (Abcam), and β-actin (Abcam).

CCK-8 assay

Cells with stable ERK knockdown or NEK2 overexpression were seeded in 96-well plates at a density of 1000 cells per well. After incubation for 24, 48, and 72 h, CCK-8 reagent (Dojindo) was added at a concentration of 10% to each well, and after incubation for 1 h, the optical density (OD) was measured at 490 nm. Experiments were performed in triplicate.

EdU incorporation assay

EdU incorporation assay was performed with an EdU Apollo 488 *in vitro* imaging kit (RiboBio). One thousand cells were seeded into each well of a 96-well plate. After incubation for 24 h, the medium was changed to a medium containing 20 mmol/L

Table 1 Primers used for quantitative real-time PCR

Gene	Primers (5' to 3', forward/reverse)
NEK2	TGCTTCGTGAACTGAAACATCC
	CCAGAGTCAACTGAGTCATCACT
ERK	CGGGGCATCTTCGAGATCG
	CAGAACAACGCCGTTTCAGTT
c-JUN	GTCCTCCATAAATGCCTGTTCC
	GATGCAACCCACTGACCAGAT
Cyclin D1	ACGAAGGTCTGCGCGTGTT
	CCGCTGGCCATGAACTACCT
GAPDH	GGAGCGAGATCCCTCCAAAAT
	GGCTGTTGTCATACTTCTCATGG

EdU, and incubation was continued at 37 °C for 1 h. After fixing the cells with 4% paraformaldehyde, Apollo staining was performed according to the standard instructions. Cell proliferation rates are shown as the percentage of EdU positive cells. Experiments were performed in triplicate.

Flow cytometry

When the cells in the 6-well plate reached 80% confluence, they were harvested using trypsin and fixed overnight in pre-cooled 70% ethanol. After washing, cells were incubated in a dark environment with 500 μL of PI/RNase staining buffer (BD Pharmingen) for 15 min. Cell cycle changes were detected and analyzed using the FACSCalibur system (BD Biosciences). Experiments were performed in triplicate.

Statistical analysis

Data were analyzed using GraphPad Prism 5 (La Jolla, CA, United States). The chi-square test or Fisher's exact test was used to determine the significance of categorical data. The Student's *t*-test or one-way ANOVA was used to determine the significance of the mean. Differences were considered statistically significant at $P < 0.05$ ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$).

RESULTS

Expression of NEK2 is significantly up-regulated in GC

The amplification of NEK2 has an oncogene effect in various types of tumors, regulating the process of tumor proliferation and invasion. To clarify the expression pattern of NEK2 in human GC, we searched the "Oncomine" database for GC data published by different researchers. It was surprised to find that NEK2 expression in GC tissues was significantly higher compared to that in normal tissues (Figure 1A). To confirm the high level of NEK2 expression in GC, we detected the NEK2 expression level in 30 pairs of GC and adjacent tissues. The results showed that the NEK2 mRNA level in GC tissues was significantly higher than that in non-cancerous tissues (Figure 1B).

In addition, in order to understand whether the high expression of NEK2 has an effect on the progression of GC, we divided 30 human GC samples into three grades (I, II, and III-IV) according to the WHO criteria for clinical GC tumors to evaluate the expression pattern of NEK2 in various stages of GC. Interestingly, high-grade tumors (Grade III-IV) showed a higher NEK2 expression level than low-grade tumors (Figure 1C), giving a suggestion that NEK2 expression is gradually increasing in the progression of GC. These results indicated that the progressively up-regulated expression of NEK2 may be a tumor-promoting regulator in the progression of GC.

To further verify that NEK2 is up-regulated in GC, the mRNA and protein expression levels of NEK2 were detected in four gastric tumor cell lines (AGS, MGC803, BGC823, and SGC7901) and gastric epithelial cell line (GES1). In the four GC cell lines, both the mRNA and protein expression levels of NEK2 were higher than those in the normal gastric epithelial cell line (Figure 1D and E), which is consistent with the expression pattern of NEK2 in the tissues. Taken together, these data can confirm that NEK2 expression shows a dynamic up-regulation pattern in GC tissues, and NEK2 plays a potential tumor-promoting role in the pathological process of GC.

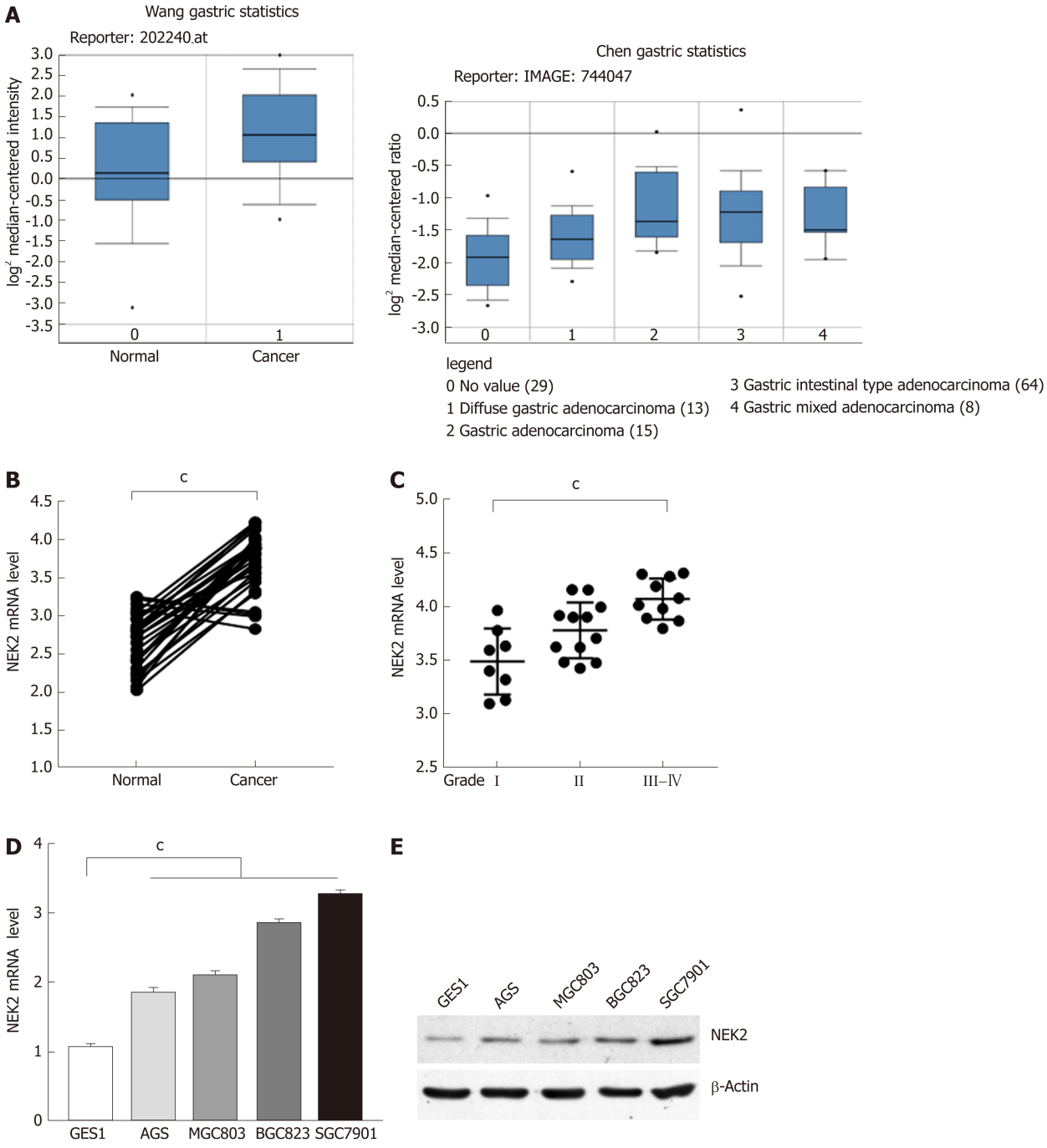


Figure 1 Expression of NIMA related kinase 2 in tissues and cells of gastric cancer. A: Relative mRNA expression level of NIMA related kinase 2 (NEK2) in gastric cancer and normal tissues (datasets from Oncomine). B: Relative mRNA expression level of NEK2 analyzed by quantitative real-time PCR in 30 pairs of human gastric cancer tissues (Cancer) and adjacent tissues (Normal). (Paired *t*-test, $P < 0.001$). C: The mRNA expression level of NEK2 in different grades of cancer samples. Data represents the mean ± SD of three replicates. D: Relative NEK2 mRNA expression level in four gastric cancer cell lines compared to gastric mucosal epithelial cell line (GES1). E: Relative NEK2 protein expression level in different gastric cancer cell lines. β-actin was used as the loading control. NEK2: NIMA related kinase 2.

Combined NEK2 and ERK expression may predict overall survival in patients with GC

Research has shown that there is a correlation between the activation of ERK1/2 and NEK2 during mitosis[29]. In order to further elucidate the molecular mechanism of the correlation of ERK1/2 and NEK2, we tried to detect the correlation of ERK1/2 and NEK2 expression in GC. First, we found that NEK2 and ERK had strong co-expression relationships in GC based on the TCGA database (Figure 2A). We next examined the ERK expression level in GC tissues, and the result showed that ERK was significantly up-regulated in GC tissue relative to normal tissue (Figure 2B). In addition, in order to confirm the expression relationship between NEK2 and ERK in GC, we further examined the expression relationship between NEK2 and ERK in clinical samples. The

RT-qPCR result showed that NEK2 was positively correlated with ERK in GC tissues in terms of the expression level (Spearman, $r = 0.594$, $P < 0.05$) (Figure 2C). Collectively, all results suggest a correlation in expression patterns between NEK2 and ERK in GC.

Although the abnormal NEK2 expression is related to the therapeutic effect and prognosis in many cancers, the prognostic significance of NEK2 in GC patients remains unclear. We first classified 30 GC tissues into different groups according the expression of NEK2 and ERK (Figure 2D). The results showed that 56.67% (17/30) of the 30 GC samples showed strong NEK2 immunostaining, while in the high NEK2 expression samples, only 23.53% (4/17) of the tumors showed low ERK expression. In the samples with low expression of NEK2 (43.33%, 13/30), only five samples exhibited high REK expression. Whatever, the $\chi 2$ test revealed a significant expression relation between ERK and NEK2 ($P = 0.035$, Figure 2E). Analysis of the expression of NEK2 and ERK using the Kaplan-Meier method in GC patients indicated that patients with low NEK2 and low ERK expression levels had better overall survival than patients with high NEK2 and high ERK levels (Figure 3D). The above results suggest that co-expression of NEK2 and ERK in tissues may be involved in the pathological process of GC and significantly affect the prognosis of patients with GC.

NEK2 promotes the activation of ERK/MAPK signaling in GC

To elucidate the underlying molecular mechanisms by which NEK2 affects the pathogenesis of GC and the prognosis of patients with GC, we analyzed the effect of NEK2 on MEK/ERK signaling. Cell proliferation-related gene expression patterns were examined in BGC823 and SGC7901 cells after NEK2 silencing. We found that, when NEK2 expression in BGC823 and SGC7901 cells was significantly reduced (Figure 4B), the expression of key genes regulating cell proliferation was significantly down-regulated, including c-JUN and cyclin D1, which are considered to be the downstream molecule of the ERK/MAPK signaling pathway (Figure 4A).

In addition, NEK2 silencing resulted in decreased levels of c-JUN and ERK phosphorylation, as revealed by Western blot analysis, although c-JUN and ERK did not change significantly at the protein level. And grayscale comparison showed their phosphorylation levels were significantly reduced (Figure 4B and C). In addition, cyclin D1 protein expression was significantly reduced after down-regulation of NEK2 expression in BGC823 and SGC7901 cells (Figure 4B). These results revealed that the expression of cyclin D1 can be regulated by NEK2 through the ERK/c-JUN pathway in GC.

NEK2 promotes cell viability, proliferation, and cell cycle progression in GC via ERK/MAPK signaling

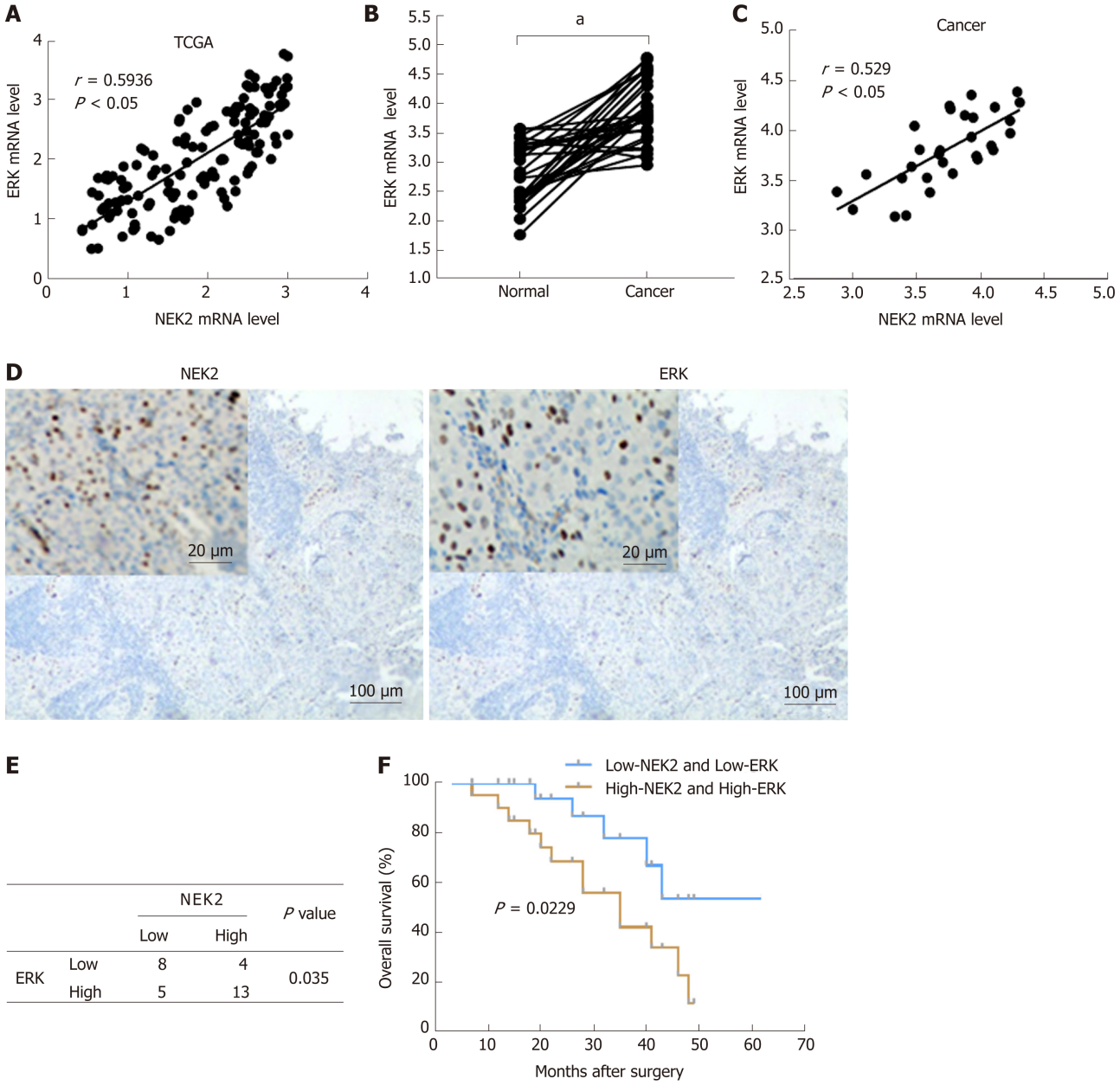
To evaluate whether NEK2 regulates potential phenotypic changes in GC cells *via* the ERK/c-JUN/cyclin D1 pathway, we used shRNA to establish BGC823 and SGC7901 cells with stable ERK knockdown. We found that cyclin D1 was significantly reduced both at the mRNA and protein levels in the NEK2 knockdown cell line compared to control cells (Figure 3A and B), while overexpression of NEK2 rescued the decreased cyclin D1 expression caused by ERK knockdown.

To explore how NEK2 mediates cell viability in GC, the CCK-8 assay was used to detect the viability of GC cells in BGC823 and SGC7901 cell lines with ERK knockdown. CCK8 assay showed that down-regulation of ERK significantly reduced the proliferation of BGC823 and SGC7901 cells after cell seeding, but cell viability was restored after stably expressing NEK2 in ERK-silenced BGC823 and SGC7901 cells (Figure 3C and D).

We next investigated the effect of silencing NEK2 on cell cycle progression by flow cytometry. Cell cycle distribution analysis showed that ERK silencing resulted in cell cycle arrest in the G0/G1 phase (Figure 4E and F), and the EdU incorporation assay showed that ERK silencing resulted in a decrease in the percentage of S phase cells, whereas both the cell cycle and DNA synthesis in BGC823 and SGC7901 cells were restored to some extent after overexpression of NEK2 (Figure 4G and H). Altogether, these data indicate that NEK2 can regulate GC cell cycle progression and cell proliferation *via* the ERK/c-JUN/cyclin D1 axis.

DISCUSSION

Previous studies have indicated that, unlike other kinases, NEK2 can promote cancer progression by participating in the regulation of centrosome activity during mitosis, leading to abnormal activation of mitosis. Therefore, the regulation of NEK2 is mainly involved in cancer during the advanced stage[30-32]. In the development of cancer at



		NEK2		*P* value
		Low	High	
ERK	Low	8	4	0.035
	High	5	13	

Figure 2 Combined NIMA related kinase 2 and ERK expression may predict overall survival in patients with gastric cancer. A: ERK co-expressed with NIMA related kinase 2 (NEK2) in gastric cancer (datasets from TCGA). B: Relative mRNA expression level of ERK analyzed by quantitative real-time PCR in 30 pairs of human gastric cancer tissues. C: ERK expression is significantly correlated to NEK2 expression in human gastric cancer tissues. D: Immunohistochemistry analysis of NEK2 and ERK expression in serial gastric cancer samples. E: Correlation between NEK2 and ERK expression. F: Overall survival of patients with gastric cancer calculated using Kaplan–Meier analysis according to the staining level. NEK2: NIMA related kinase 2.

early stages, for example, NEK2 promotes tumor cell proliferation and participates in the invasion of tumor cells to epithelial tissues[9,16]. It is speculated that enhanced activity of centrosomes is a feature of advanced cell carcinogenesis, driving mutations to progeny cells and providing a source of genetic variation in tumorigenesis[33].

In this study, we for the first time showed that NEK2 overexpression promoted the activation of c-JUN and the up-regulation of cyclin D1 through the abnormally activated ERK/MAPK pathway in GC. We also found that NEK2 overexpression promoted the cell cycle progression in GC cell lines and participated in the regulation of proliferation of GC cells. In human myeloma, NEK2 promotes myeloma resistance *via* activating the ERK pathway, indirectly supporting our conclusion[34-36]. Thus, our results suggest that NEK2 may have a new and important role in tumor progression.

In many mammalian cell types, the ERK pathway can integrate cellular external stimulation signals into cells, acting as a significant regulator that mediates cell growth and proliferation[37,38]. It is well known that uncontrolled growth or malignant proliferation is a necessary and initial step in the development of tumors[39,40]. Furthermore, it has been shown that abnormal NEK2 expression obviously affected

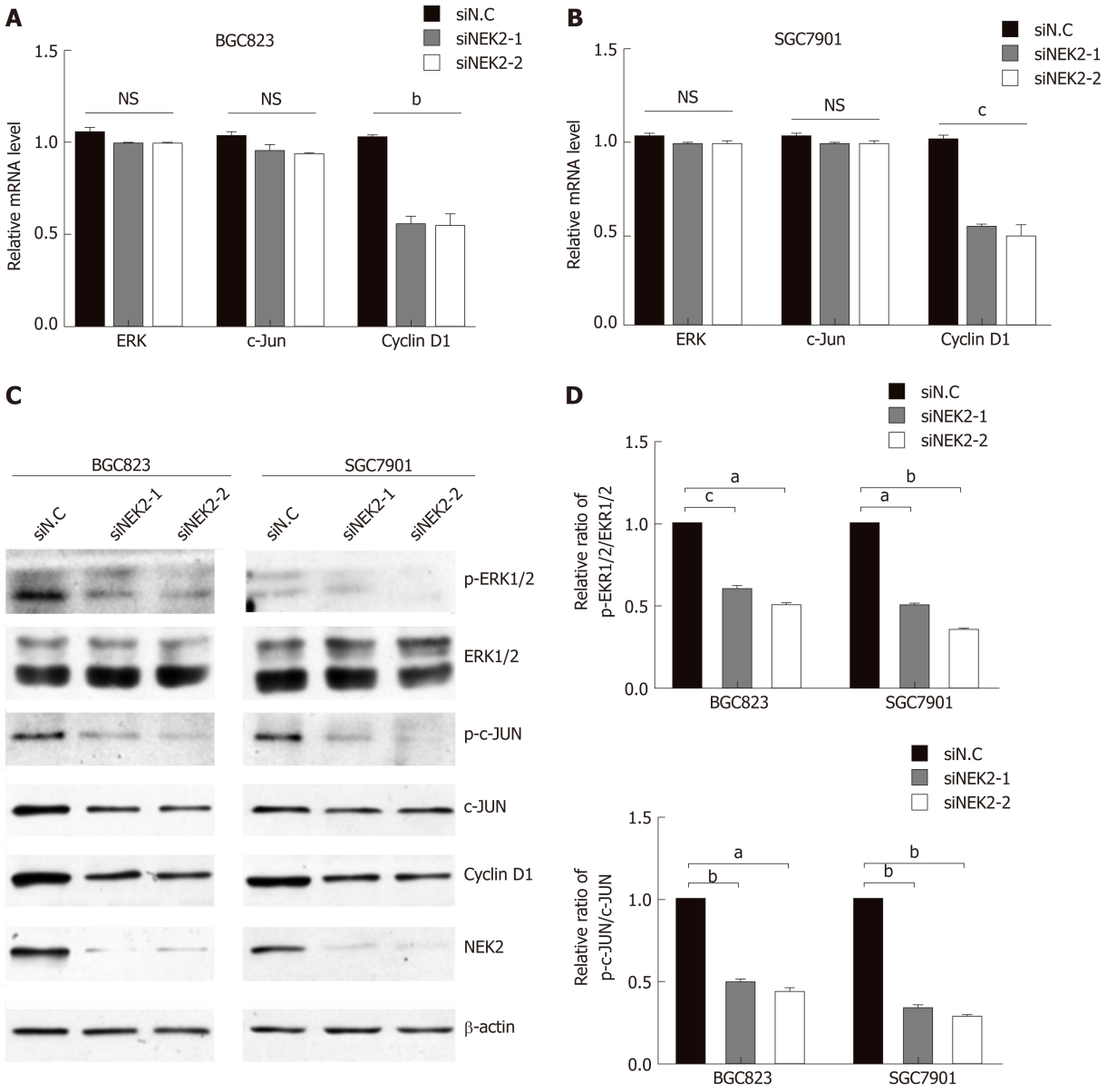
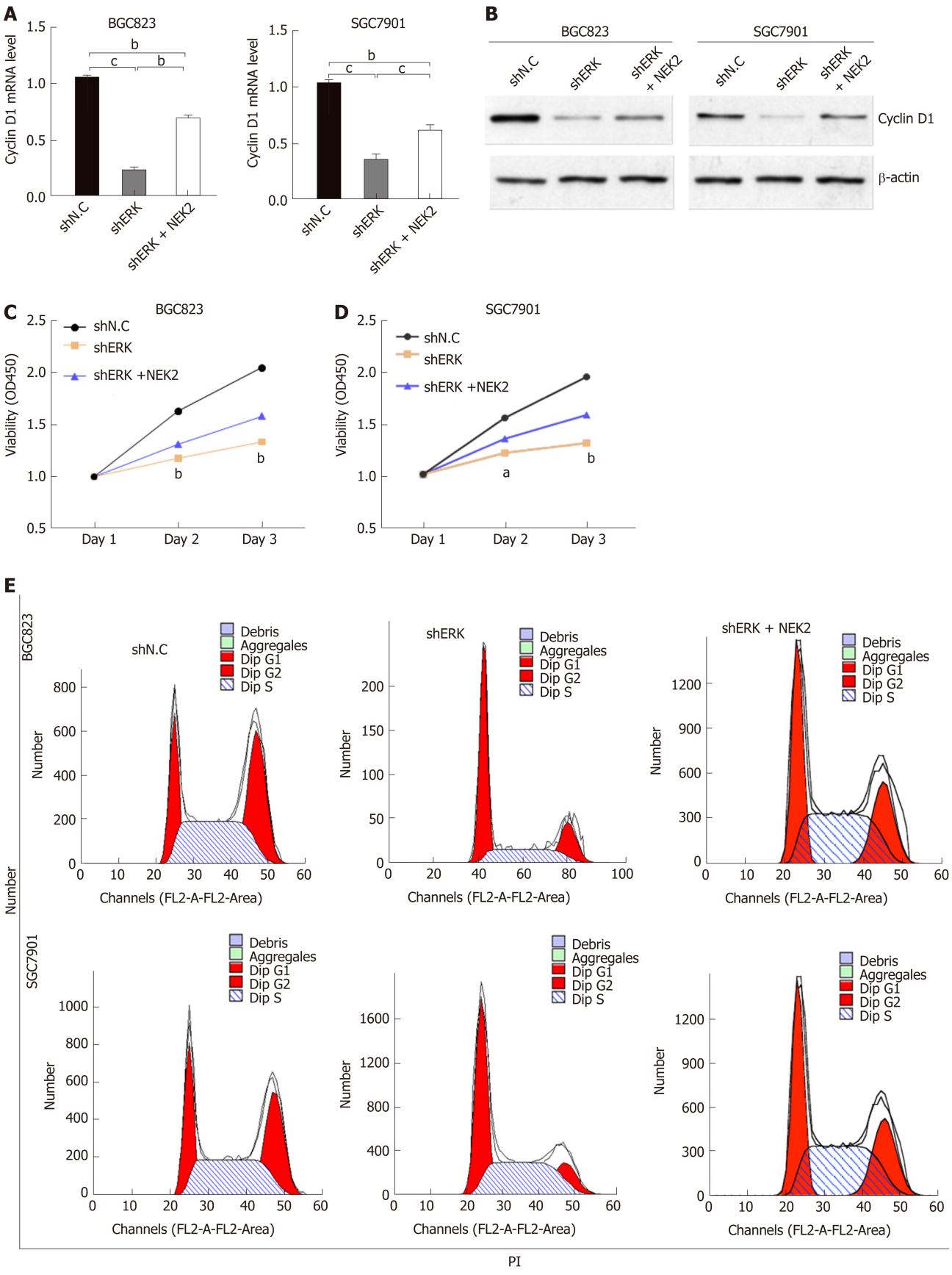
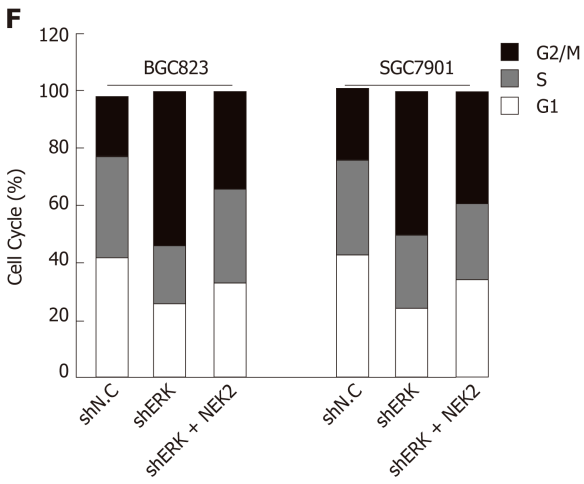


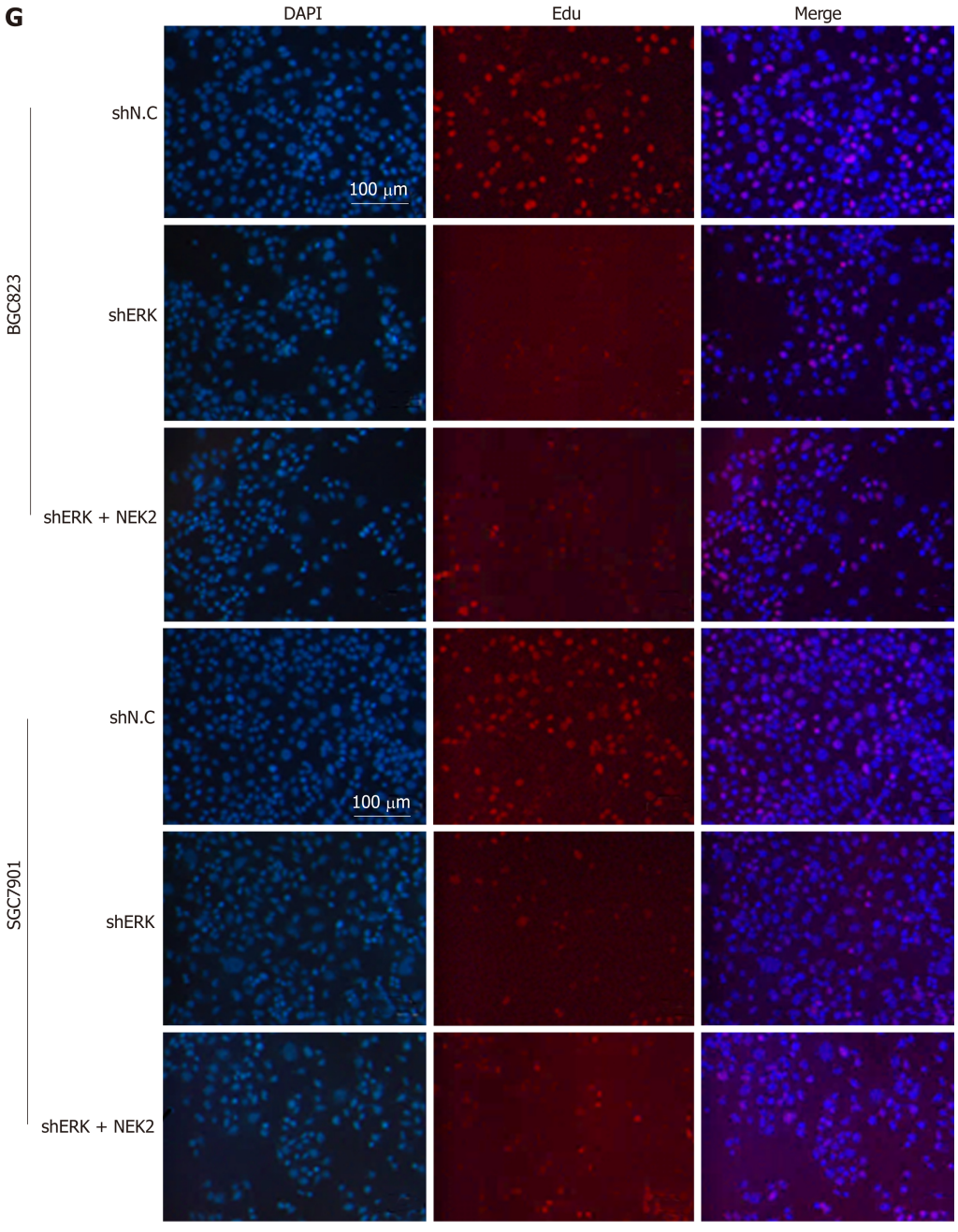
Figure 3 NIMA related kinase 2 mediates the activation of ERK/MAPK signaling. A and B: Relative mRNA expression of ERK/MAPK signaling key factors analyzed by quantitative real-time PCR in NIMA related kinase 2 (NEK2) silenced BGC823 and SGC7901 cell lines and negative controls. C: Western blot analysis of ERK/MAPK signaling key factors (ERK, c-JUN, and cyclin D1) and their phosphorylated forms (p-ERK and p-c-JUN) in NEK2 silenced BGC823 and SGC7901 cell lines and negative controls. D: Relative phosphorylation levels of ERK and c-JUN in C were calculated. β-actin was used as the internal control. Values are presented as the mean ± SEM. NEK2: NIMA related kinase 2.

the process of mitosis of breast tumor cells, and in breast cancer, NEK2 overexpression was significantly associated with a poor prognosis, indicating that NEK2 may be an ideal target for therapy[9,41]. However, in our study, we demonstrated that NEK2 was highly expressed in GC and the up-regulated expression of NEK2 was associated with the activity of the ERK/MAPK pathway. NEK2 can promote phosphorylation of ERK and further promote cyclin D1 expression through the ERK/c-JUN pathway, regulating the mitosis of GC cells. In addition, the combined and elevated expression of NEK2 and ERK was found to be significantly associated with a poor prognosis in patients with GC, indicating that NEK2 is an essential factor that mediates the transmission of important signals in GC. Therefore, NEK2 has the potential to become an ideal target for GC therapy[41,42].

In conclusion, our study reveals a novel mechanism by which NEK2 promotes the growth of GC cells by activating the ERK/MAPK pathway. Although we have demonstrated that NEK2 can regulate the phosphorylation of c-JUN and ERK and cell cycle progression in GC cells, there may be other targets that may also affect cancer cell proliferation. Nonetheless, our finding that NEK2 targets the ERK/c-JUN/cyclin D1 pathway provides new insights into NEK2 function and reveals that NEK2 may be a potential target for developing optimal treatments for GC patients.


A
BGC823
SGC7901
Cyclin D1 mRNA level
shN.C
shERK
shERK + NEK2
B
BGC823
SGC7901
Cyclin D1
β-actin
C
BGC823
Viability (OD450)
shN.C
shERK
shERK +NEK2
Day 1
Day 2
Day 3
D
SGC7901
E
BGC823
SGC7901
Debris
Aggregales
Dip G1
Dip G2
Dip S
Number
Channels (FL2-A-FL2-Area)
PI


F
120
100
80
60
40
20
0
Cell Cycle (%)
BGC823
SGC7901
G2/M
S
G1
shN.C
shERK
shERK + NEK2
shN.C
shERK
shERK + NEK2


G
DAPI
Edu
Merge
BGC823
shN.C
100 μm
shERK
shERK + NEK2
SGC7901
shN.C
100 μm
shERK
shERK + NEK2

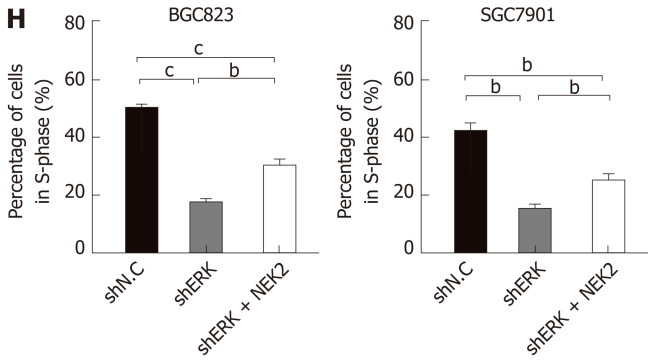


Figure 4 NIMA related kinase 2 promotes gastric cancer cell viability, proliferation, and cell cycle progression *via* ERK/MAPK signaling. A and B: Relative mRNA and protein expression of cyclin D1 in cell lines with ERK silencing and NEK2 overexpression. C and D: CCK8 assay showing cell viability in BGC823 and SGC7901 cell lines and negative controls. E: Flow cytometry analysis of cell cycle distribution in BGC823 and SGC7901 cell lines. F: Quantification of the data shown in E (n = 3). G: EdU incorporation assay showing the percentage of cells in S-phase. H: Quantification of the data shown in G (n = 3). NEK2: NIMA related kinase 2.

ARTICLE HIGHLIGHTS

Research background

NIMA related kinase 2 (NEK2) is closely related to mitosis, and it is currently considered to be overexpressed frequently in many poorly prognostic cancers. However, the effect of up-regulated NEK2 expression on cellular signaling in tumors, such as gastric cancer (GC), is confusing.

Research motivation

To explore the treatment of GC and improve patient survival.

Research objectives

To determine the role of the up-regulation of NEK2 in GC.

Research methods

To investigate the pathological significance of NEK2 in GC, the expression of NEK2 in GC was investigated based on the "Oncomain" database and compared between 30 pairs of cancer and adjacent tissues. The co-expression of NEK2 and ERK in GC was analyzed based on the TCGA database and confirmed in clinical samples by quantitative real-time PCR (qRT-PCR), and the survival curve was also plotted. Western blot or qRT-PCR was used to analyze the effect of NEK2 on the phosphorylation levels of ERK and c-JUN in BGC823 and SGC7901 cells, and the expression of the downstream effector cyclin D1. Furthermore, CCK8, EdU incorporation assay, and flow cytometry were used to detect the proliferative of BGC823 and SGC7901 cell lines with stable silencing of ERK.

Research results

In this study, we found that NEK2 was significantly up-regulated in human GC tissues. In addition, ERK was significantly associated with NEK2 expression in human clinical specimens, and combined overexpression of NEK2 and ERK potentially forecasted a poor prognosis and survival in GC patients. NEK2 knockdown in GC cells inhibited ERK and c-JUN phosphorylation and reduced transcription of cyclin D1. More interestingly, NEK2 can rescue the inhibition of cellular viability, proliferation, and cell cycle progression due to ERK knockdown.

Research conclusions

NEK2 plays a carcinogenic role in the malignant proliferation of GC cells *via* the ERK/MAPK signaling, which is important for treating GC and improving patient survival.

Research perspectives

Future research may further reveal the mechanism of action of NEK2 to enhance the sensitivity of cancer cells and promote its application in anti-cancer treatments. And the identification of the molecular pathway related to ERK/MAPK signaling may further elucidate the underlying mechanism.

REFERENCES

1 **Strand MS**, Lockhart AC, Fields RC. Genetics of Gastric Cancer. *Surg Clin North Am* 2017; **97**: 345-370 [PMID: 28325191 DOI: 10.1016/j.suc.2016.11.009]

2 **Antoni S**, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F. Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. *Eur Urol* 2017; **71**: 96-108 [PMID: 27370177 DOI: 10.1016/j.eururo.2016.06.010]

3 **Miller KD**, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin* 2016; **66**: 271-289 [PMID: 27253694 DOI: 10.3322/caac.21349]
4 **Mihmanli M**, Ilhan E, Idiz UO, Alemdar A, Demir U. Recent developments and innovations in gastric cancer. *World J Gastroenterol* 2016; **22**: 4307-4320 [PMID: 27158199 DOI: 10.3748/wjg.v22.i17.4307]
5 **Lansdorp-Vogelaar I**, Kuipers EJ. Screening for gastric cancer in Western countries. *Gut* 2016; **65**: 543-544 [PMID: 26611232 DOI: 10.1136/gutjnl-2015-310356]
6 **Liu Z**, Gao P, Liu S, Zheng G, Yang J, Sun L, Hong L, Fan D, Zhang H, Feng F. Tumor volume increases the predictive accuracy of prognosis for gastric cancer: A retrospective cohort study of 3409 patients. *Oncotarget* 2017; **8**: 18968-18978 [PMID: 28145885 DOI: 10.18632/oncotarget.14859]
7 **Schipper DL**, Wagenmans MJ, Peters WH, Wagener DJ. Significance of cell proliferation measurement in gastric cancer. *Eur J Cancer* 1998; **34**: 781-790 [PMID: 9797687]
8 **Rugge M**, Genta RM, Di Mario F, El-Omar EM, El-Serag HB, Fassan M, Hunt RH, Kuipers EJ, Malfertheiner P, Sugano K, Graham DY. Gastric Cancer as Preventable Disease. *Clin Gastroenterol Hepatol* 2017; **15**: 1833-1843 [PMID: 28532700 DOI: 10.1016/j.cgh.2017.05.023]
9 **Zhou W**, Yang Y, Xia J, Wang H, Salama ME, Xiong W, Xu H, Shetty S, Chen T, Zeng Z, Shi L, Zangari M, Miles R, Bearss D, Tricot G, Zhan F. NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers. *Cancer Cell* 2013; **23**: 48-62 [PMID: 23328480 DOI: 10.1016/j.ccr.2012.12.001]
10 **Cappello P**, Blaser H, Gorrini C, Lin DC, Elia AJ, Wakeham A, Haider S, Boutros PC, Mason JM, Miller NA, Youngson B, Done SJ, Mak TW. Role of Nek2 on centrosome duplication and aneuploidy in breast cancer cells. *Oncogene* 2014; **33**: 2375-2384 [PMID: 23708664 DOI: 10.1038/onc.2013.183]
11 **Hayward DG**, Clarke RB, Faragher AJ, Pillai MR, Hagan IM, Fry AM. The centrosomal kinase Nek2 displays elevated levels of protein expression in human breast cancer. *Cancer Res* 2004; **64**: 7370-7376 [PMID: 15492258 DOI: 10.1158/0008-5472.Can-04-0960]
12 **Ha Kim Y**, Yeol Choi J, Jeong Y, Wolgemuth DJ, Rhee K. Nek2 localizes to multiple sites in mitotic cells, suggesting its involvement in multiple cellular functions during the cell cycle. *Biochem Biophys Res Commun* 2002; **290**: 730-736 [PMID: 11785960 DOI: 10.1006/bbrc.2001.6212]
13 **Fry AM**, O'Regan L, Sabir SR, Bayliss R. Cell cycle regulation by the NEK family of protein kinases. *J Cell Sci* 2012; **125**: 4423-4433 [PMID: 23132929 DOI: 10.1242/jcs.111195]
14 **Fry AM**. The Nek2 protein kinase: a novel regulator of centrosome structure. *Oncogene* 2002; **21**: 6184-6194 [PMID: 12214248 DOI: 10.1038/sj.onc.1205711]
15 **Hames RS**, Wattam SL, Yamano H, Bacchieri R, Fry AM. APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *EMBO J* 2001; **20**: 7117-7127 [PMID: 11742988 DOI: 10.1093/emboj/20.24.7117]
16 **Kokuryo T**, Senga T, Yokoyama Y, Nagino M, Nimura Y, Hamaguchi M. Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. *Cancer Res* 2007; **67**: 9637-9642 [PMID: 17942892 DOI: 10.1158/0008-5472.Can-07-1489]
17 **Wang S**, Li W, Liu N, Zhang F, Liu H, Liu F, Liu J, Zhang T, Niu Y. Nek2A contributes to tumorigenic growth and possibly functions as potential therapeutic target for human breast cancer. *J Cell Biochem* 2012; **113**: 1904-1914 [PMID: 22234886 DOI: 10.1002/jcb.24059]
18 **Chen Y**, Riley DJ, Zheng L, Chen PL, Lee WH. Phosphorylation of the mitotic regulator protein Hec1 by Nek2 kinase is essential for faithful chromosome segregation. *J Biol Chem* 2002; **277**: 49408-49416 [PMID: 12386167 DOI: 10.1074/jbc.M207069200]
19 **Richardson AL**, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* 2006; **9**: 121-132 [PMID: 16473279 DOI: 10.1016/j.ccr.2006.01.013]
20 **Lee J**, Gollahon L. Nek2-targeted ASO or siRNA pretreatment enhances anticancer drug sensitivity in triplenegative breast cancer cells. *Int J Oncol* 2013; **42**: 839-847 [PMID: 23340795 DOI: 10.3892/ijo.2013.1788]
21 **Davis RJ**. Signal transduction by the JNK group of MAP kinases. *Cell* 2000; **103**: 239-252 [PMID: 11057897]
22 **Wagner EF**, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 2009; **9**: 537-549 [PMID: 19629069 DOI: 10.1038/nrc2694]
23 **Weston CR**, Davis RJ. The JNK signal transduction pathway. *Curr Opin Genet Dev* 2002; **12**: 14-21 [PMID: 11790549]
24 **Pearson G**, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; **22**: 153-183 [PMID: 11294822 DOI: 10.1210/edrv.22.2.0428]
25 **Edmunds JW**, Mahadevan LC. MAP kinases as structural adaptors and enzymatic activators in transcription complexes. *J Cell Sci* 2004; **117**: 3715-3723 [PMID: 15286173 DOI: 10.1242/jcs.01346]
26 **Li S**, Ma YM, Zheng PS, Zhang P. GDF15 promotes the proliferation of cervical cancer cells by phosphorylating AKT1 and Erk1/2 through the receptor ErbB2. *J Exp Clin Cancer Res* 2018; **37**: 80 [PMID: 29636108 DOI: 10.1186/s13046-018-0744-0]
27 **Lin CL**, Lee CH, Chen CM, Cheng CW, Chen PN, Ying TH, Hsieh YH. Protodioscin Induces Apoptosis Through ROS-Mediated Endoplasmic Reticulum Stress via the JNK/p38 Activation Pathways in Human Cervical Cancer Cells. *Cell Physiol Biochem* 2018; **46**: 322-334 [PMID: 29590661 DOI: 10.1159/000488433]
28 **Yan L**, Liu X, Yin A, Wei Y, Yang Q, Kong B. Huaier aqueous extract inhibits cervical cancer cell proliferation via JNK/p38 pathway. *Int J Oncol* 2015; **47**: 1054-1060 [PMID: 26201539 DOI: 10.3892/ijo.2015.3094]
29 **Di Agostino S**, Rossi P, Geremia R, Sette C. The MAPK pathway triggers activation of Nek2 during chromosome condensation in mouse spermatocytes. *Development* 2002; **129**: 1715-1727 [PMID: 11923207]
30 **Tsunoda N**, Kokuryo T, Oda K, Senga T, Yokoyama Y, Nagino M, Nimura Y, Hamaguchi M. Nek2 as a novel molecular target for the treatment of breast carcinoma. *Cancer Sci* 2009; **100**: 111-116 [PMID: 19038001 DOI: 10.1111/j.1349-7006.2008.01007.x]
31 **Hayward DG**, Fry AM. Nek2 kinase in chromosome instability and cancer. *Cancer Lett* 2006; **237**: 155-166 [PMID: 16084011 DOI: 10.1016/j.canlet.2005.06.017]
32 **Carter SL**, Eklund AC, Kohane IS, Harris LN, Szallasi Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* 2006; **38**:

1043-1048 [PMID: 16921376 DOI: 10.1038/ng1861]
33 **Piao T**, Luo M, Wang L, Guo Y, Li D, Li P, Snell WJ, Pan J. A microtubule depolymerizing kinesin functions during both flagellar disassembly and flagellar assembly in Chlamydomonas. *Proc Natl Acad Sci USA* 2009; **106**: 4713-4718 [PMID: 19264963 DOI: 10.1073/pnas.0808671106]
34 **Wang X**, Zhou YX, Qiao W, Tominaga Y, Ouchi M, Ouchi T, Deng CX. Overexpression of aurora kinase A in mouse mammary epithelium induces genetic instability preceding mammary tumor formation. *Oncogene* 2006; **25**: 7148-7158 [PMID: 16715125 DOI: 10.1038/sj.onc.1209707]
35 **Spalluto C**, Wilson DI, Hearn T. Nek2 localises to the distal portion of the mother centriole/basal body and is required for timely cilium disassembly at the G2/M transition. *Eur J Cell Biol* 2012; **91**: 675-686 [PMID: 22613497 DOI: 10.1016/j.ejcb.2012.03.009]
36 **Kobayashi T**, Dynlacht BD. Regulating the transition from centriole to basal body. *J Cell Biol* 2011; **193**: 435-444 [PMID: 21536747 DOI: 10.1083/jcb.201101005]
37 **Orton RJ**, Sturm OE, Vyshemirsky V, Calder M, Gilbert DR, Kolch W. Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway. *Biochem J* 2005; **392**: 249-261 [PMID: 16293107 DOI: 10.1042/bj20050908]
38 **Schulze WX**, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* 2005; **1**: 2005.0008 [PMID: 16729043 DOI: 10.1038/msb4100012]
39 **Zarich N**, Oliva JL, Martínez N, Jorge R, Ballester A, Gutiérrez-Eisman S, García-Vargas S, Rojas JM. Grb2 is a negative modulator of the intrinsic Ras-GEF activity of hSos1. *Mol Biol Cell* 2006; **17**: 3591-3597 [PMID: 16760435 DOI: 10.1091/mbc.e05-12-1104]
40 **Meloche S**, Pouysségur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene* 2007; **26**: 3227-3239 [PMID: 17496918 DOI: 10.1038/sj.onc.1210414]
41 **Gschwind A**, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev Cancer* 2004; **4**: 361-370 [PMID: 15122207 DOI: 10.1038/nrc1360]
42 **Wu G**, Qiu XL, Zhou L, Zhu J, Chamberlin R, Lau J, Chen PL, Lee WH. Small molecule targeting the Hec1/Nek2 mitotic pathway suppresses tumor cell growth in culture and in animal. *Cancer Res* 2008; **68**: 8393-8399 [PMID: 18922912 DOI: 10.1158/0008-5472.CAN-08-1915]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2911-2923

DOI: 10.3748/wjg.v25.i23.2911

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Proteomics of the mediodorsal thalamic nucleus of rats with stress-induced gastric ulcer

Sheng-Nan Gong, Jian-Ping Zhu, Ying-Jie Ma, Dong-Qin Zhao

ORCID number: Sheng-Nan Gong (0000-0002-0522-8536); Jian-Ping Zhu (0000-0002-2057-674X); Ying-Jie Ma (0000-0003-1216-008X); Dong-Qin Zhao (0000-0002-9861-7038).

Author contributions: Zhao DQ and Zhu JP designed the experiments; Ma YJ and Gong SN performed the experiments; Zhao DQ performed mass spectrometry analysis and wrote the manuscript; Gong SN analyzed the protemoics data; Zhu JP analyzed the GM and EI data; Ma YJ analyzed the WB data; all the authors approved the final version of the manuscript for publication.

Supported by National Natural Science Foundation of China, No. 31501861; and Natural Science Foundation of Shandong Province, China, No. ZR2015CM013.

Institutional animal care and use committee statement: All procedures were approved by the Institutional Animal Care and Use Committee of Shandong Normal University (Jinan, People's Republic of China; No. AEECSDNU2018003).

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Sheng-Nan Gong, Jian-Ping Zhu, Ying-Jie Ma, Dong-Qin Zhao, College of Life Sciences, Shandong Normal University, Jinan 250014, Shandong Province, China

Corresponding author: Dong-Qin Zhao, PhD, Associate Professor, College of Life Sciences, Shandong Normal University, No. 88, East Wenhua Road, Lixia District, Jinan 250014, Shandong Province, China. zhaodq@sdnu.edu.cn
Telephone: +86-531-86180745

Abstract

BACKGROUND

Stress-induced gastric ulcer (SGU) is one of the most common visceral complications after trauma. Restraint water-immersion stress (RWIS) can cause serious gastrointestinal dysfunction and has been widely used to study the pathogenesis of SGU to identify medications that can cure the disease. The mediodorsal thalamic nucleus (MD) is the centre integrating visceral and physical activity and contributes to SGU induced by RWIS. Hence, the role of the MD during RWIS needs to be studied.

AIM

To screen for differentially expressed proteins in the MD of the RWIS rats to further elucidate molecular mechanisms of SGU.

METHODS

Male Wistar rats were selected randomly and divided into two groups, namely, a control group and an RWIS group. Gastric mucosal lesions of the sacrificed rats were measured using the erosion index and the proteomic profiles of the MD were generated through isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two-dimensional liquid chromatography and tandem mass spectrometry. Additionally, iTRAQ results were verified by Western blot analysis.

RESULTS

A total of 2853 proteins were identified, and these included 65 dysregulated (31 upregulated and 34 downregulated) proteins (fold change ratio ≥ 1.2). Gene Ontology (GO) analysis showed that most of the upregulated proteins are primarily related to cell division, whereas most of the downregulated proteins are related to neuron morphogenesis and neurotransmitter regulation. Ingenuity Pathway Analysis revealed that the dysregulated proteins are mainly involved in the neurological disease signalling pathways. Furthermore, our results indicated that glycogen synthase kinase-3 beta might be related to the central mechanism

Manuscript source: Unsolicited manuscript

Received: January 27, 2019
Peer-review started: January 27, 2019
First decision: March 20, 2019
Revised: May 8, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019
Published online: June 21, 2019

P-Reviewer: Ierardi E, Liu HK
S-Editor: Cui LJ
L-Editor: Wang TQ
E-Editor: Ma YJ

through which RWIS gives rise to SGU.

CONCLUSION
Quantitative proteomic analysis elucidated the molecular targets associated with the production of SGU and provides insights into the role of the MD. The underlying molecular mechanisms need to be further dissected.

Key words: Mediodorsal thalamic nucleus; Proteome; Restraint water-immersion stress; Stress-induced gastric ulcer; Glycogen synthase kinase-3 beta

Core tip: Quantitative proteomic analysis was used to screen for differentially expressed proteins in the mediodorsal thalamic nucleus of rats subjected to restraint water-immersion stress. A total of 65 dysregulated proteins were identified, which are mainly involved in the neurological disease signalling pathways. Meanwhile, the 31 upregulated proteins are primarily related to cell division, whereas the 34 downregulated proteins are related to neuron morphogenesis and neurotransmitter regulation. Furthermore, glycogen synthase kinase-3 beta might be related to the central mechanism through which restraint water-immersion stress gives rise to stress-induced gastric ulcer (SGU). Proteomic analysis elucidated the nervous system molecular targets of SGU.

Citation: Gong SN, Zhu JP, Ma YJ, Zhao DQ. Proteomics of the mediodorsal thalamic nucleus of rats with stress-induced gastric ulcer. *World J Gastroenterol* 2019; 25(23): 2911-2923
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2911.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2911

INTRODUCTION

Stress can cause behaviour, immune, and neural responses, and intense and persistent stress responses could lead to a wide range of physiological and psychological dysfunctions in the body[1-3]. Restraint water-immersion stress (RWIS) can cause serious gastrointestinal dysfunction and has been widely used to study the underlying molecular mechanisms of stress-induced gastric ulcer (SGU) and to screen potential curative drugs[4,5]. The primary central mechanism through which brainstem circuits regulate gastric function under normal physiological conditions has been fundamentally clarified[6-8], and the peripheral regulatory mechanism of gastric ulcers induced by RWIS has also drawn much attention[9-12]. Previous studies have reported that neuronal hyperactivity of vagal parasympathetic efferents, which mostly originate in the primary gastrointestinal control centre of the central medulla (mainly in the dorsal motor nucleus of the vagus and partly in the nucleus ambiguous) and are further mediated by the higher central-anterior hypothalamus (mainly the paraventricular nucleus and supraoptic nucleus), leads to gastric dysfunction[13-15]. In addition, we previously found that catecholaminergic, vasopressinergic, and oxytocinergic neurons might be associated with gastric ulcers induced by RWIS[16-18]. The brain nuclei and neurotransmitters or hormones involved in RWIS are shown in Table 1[10,11,13-15,19,20]. However, we have not yet retrieved relevant papers that discuss the effects of proteomic changes on the brain of rats during exposure to RWIS.

The mediodorsal thalamic nucleus (MD) has complex connections with the prefrontal cortex and subcortical structures[21-26], and previous studies have revealed that the MD is the integration centre of visceral and physical activity and is involved in the modulation of emotion[27-31], cognition, and memory[32-36]. We previously found that the expression of Fos, which is used as a marker of neuronal activity, is significantly increased in the MD of rats after 2 h of RWIS[37]. Hence, the role of the MD during RWIS needs to be studied. A comparative proteomic analysis of RWIS-exposed and control rats might not only shed light on the role of the MD in gastrointestinal dysfunction induced by RWIS but also contribute to the detection of proteomic differences and the identification of targets for more specific therapies.

The combination of isobaric tags for relative and absolute quantitation (iTRAQ) with multidimensional liquid chromatography and tandem mass spectrometry

Table 1 Brain nuclei and neurotransmitters/hormones involved in restraint water-immersion stress

Nucleus	Neurotransmitter or hormone
Dorsal motor nucleus of the vagus[10]	Acetylcholine[19]
Nucleus ambiguous[10]	Norepinephrine[13]
Nucleus of solitary tract[10]	Oxytocin[14]
Paraventricular nucleus[10]	Arginine vasopressin[14,15]
Supraoptic nucleus[11]	Hydrogen peroxide[20]

(LC–MS/MS) analysis is one of the most sensitive proteomic technologies for detecting and quantitatively analysing low-abundance proteins, and this approach has been widely used to discover disease-specific targets and biomarkers for a variety of diseases[12,38-41]. To the best of our knowledge, an iTRAQ-based approach has been applied to study the central mechanism of gastrointestinal dysfunction induced by RWIS. To identify dysregulated proteins in the MD, which might help to further explore the molecular mechanisms and pathogenesis of SGU induced by RWIS, we quantitatively compared the proteomes of control and RWIS-exposed rats using the multiplex capability of the iTRAQ approach. The present study provided a novel understanding of the role of the MD, and the newly identified molecular differences might provide fundamental information for further study.

MATERIALS AND METHODS

Preparation of animals

Adult male Wistar rats, weiging 250-300 g and provided by the Experimental Animal Center of Shandong University, Jinan, China, were housed two per cage at 22 °C ± 2 °C under a 12 h light-12 h dark cycle with free access to food and water before the initiation of RWIS. The sxperiments were initiated at least 7 days after the animals arrived at the facility. Prior to the experiments, rats were fasted for 24 h but allowed access to tap water *ad libitum*.

Stress protocols

Twenty-four male rats, numbered from 01 to 24, were used in the stress experiments. Twelve rats, which were selected using the random number table method, were subjected to RWIS for 4 h (RWIS group), and the other rats were not subjected to RWIS (control group). RWIS was performed as previously described[16]. Experiments were performed between 8:00 morning and 12:00 afternoon. All procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Shandong Normal University (Jinan, People's Republic of China; No. AEECSDNU2018003). In addition, all efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Evaluation of gastric mucosal damage

At the end of the procedure, rats were killed through a pentobarbital-Na^+ injection (100 mg/kg BW, *i.p.*). Stomachs were removed, placed in 1% formaldehyde solution for 30 min, and incised along the greater curvature. Gastric mucosal lesions were measured using the erosion index (EI)[42].

Sample collection

After brain dissection, the MD (coordinates: 2.04-3.84 mm rostral to the obex, depth of 5.0-6.0 mm below the surface of the obex, and 1.0 mm lateral to the midline) was harvested for protein extraction (WPI, Sarasota, United States)[43]. To minimize the individual differences of rats, every three individual samples were mixed for protein extraction, and protein extracts were prepared in duplicate for each group.

Protein extraction and iTRAQ labelling

The sample was resuspended in approximately eight times volume of lysis buffer (4% SDS and 100 mM HEPES (pH = 7.6) containing protease inhibitor cocktail and PMSF). The homogenate was sonicated for 30 min on ice. After centrifugation at 25 000 *g* for 30 min at 4 °C, the supernatant was collected and stored at -80 °C. The total protein concentration was measured using the BCA Kit (Trans Gen Biotech, Beijing, China).

Equal amounts of the extracted protein were mixed according to groups and then precipitated with acetone overnight. After the protein was resuspended in triethylammonium bicarbonate (TEAB) buffer, protein quantification was done using the BCA Kit. The proteins were reduced by 5 mmol/L DTT at 56 °C for 30 min and alkylated by 10 mM IAA at room temperature for 30 min. The sample was then diluted with 50 mM ammonium bicarbonate until the concentration of urea was lower than 1 M. Trypsin (Sigma) was added to the sample at a mass ratio of 1:50 (enzyme:protein) for 12 h. iTRAQ-8plex labelling reagents (AB Sciex) were added to the peptide samples, which were then incubated at room temperature for 120 min. The reaction was stopped by the addition of water, followed by concentration of the product using SpeedVac and desalts. The purified peptides were collected and stored at -80 °C until use.

High-pH reverse-phase fractionation and LC–MS/MS detection

The peptides were fractionated on a Waters UPLC using a C 18 column (Waters BEH, Milford, United States, c18 2.1×50 mm, 1.7 μm). Peptides were eluted at a flow rate of 600 μL/min with a linear gradient of 5%−35% solvent B (acetonitrile) over 10 min, with solvent A composed of 20 mM ammonium formate with pH adjusted to 10. The absorbance at 214 nm was monitored, and a total of ten fractions were collected.

The fractions were separated by nano-HPLC (Eksigent Technologies) on a secondary reversed-phase analytical column (Eksigent, C18, 3 μm, 150 mm × 75 μm). Peptides were subsequently eluted using the following gradient conditions with phase B (98% ACN with 0.1% formic acid) from 5% to 40% B (5-65 min), 40% to 80% B (66-71 min), and 80% to 5% B (72-90 min), and total flow rate was maintained at 300 nL/min. Electrospray voltage of 2.5 kV versus the inlet of the mass spectrometer was used. The QTOF 4600 mass spectrometer was operated in information-dependent data acquisition mode to shift automatically between MS and MS/MS acquisition. MS spectra were acquired across the mass range of 350-1250 m/z. The 25 most intense precursors were selected for fragmentation per cycle with a dynamic exclusion time of 25 s.

Data processing

After product separation, all MS/MS spectra were analysed using Mascot (Matrix Science, London, United Kingdom; version 2.3.0). Mascot was set up to search the UniProt_rat database and was searched with a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 25.0 ppm. Additionally, carbamidomethylation of cysteine and the iTRAQ 8PLEX conjugation of lysine and the N-terminus were specified in Mascot as fixed modifications. Oxidation of methionine and iTRAQ 8PLEX conjugation of tyrosine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR, United States) was used to validate the MS/MS-based peptide and protein identifications. Peptide identifications were accepted at a false discovery rate (FDR) of less than 1.0% using the Scaffold Local FDR algorithm. Protein probabilities were assigned using the Protein Proph *et al*[44] gorithm. Only those proteins with greater than 90% probabilities were accepted.

Quantitative data analysis

Scaffold Q+ (version Scaffold_4.4.5, Proteome Software Inc., Portland, OR, United States) was used to quantify the iTRAQ peptide and for protein identifications. To determine quantitative changes, a ± 1.2-fold change in the MD of rats between the control and RWIS groups with $P < 0.05$, as calculated by the Student's *t*-test, was used as the threshold for categorizing upregulated and downregulated proteins.

Bioinformatic analysis

Gene Ontology (GO) annotation and pathway enrichment analysis of all the identified and differentially expressed proteins were implemented using the online tool DAVID (http://david.abcc.ncifcrf.gov/). GO annotation includes biological processes (BP), cellular components (CC), and molecular functions (MF). Ingenuity Pathway Analysis (IPA) software (version 7.5, Ingenuity System Inc., Redwood City, CA, United States; www.ingenuity.com) was used to identify the biological functions and signalling pathways of the annotated differentially expressed proteins. The GO annotations involving signalling pathways and networks were ranked based on the enrichment of the differentially expressed proteins.

Western blot analysis

The results of the proteomic experiment were verified by Western blot analysis. Protein extracts (30 μg) were separated by SDS-PAGE followed by electrotransfer onto PVDF membranes (PVDF, Millipore, Bedford, MA, United States). The membranes

were blocked with 5% non-fat dry milk in 0.01 M PBS containing 0.1% Tween-20 for 2 h at room temperature and then incubated at 4 °C overnight with the following primary antibodies: Anti-glycogen synthase kinase-3 beta (GSK3B) (mouse monoclonal, 47 kDa; 1:2000 dilution; Abcam plc ab93926, Cambridge Science Park, Cambridge, CB4 0FL, United Kingdom) and anti-synaptophysin (SYN) (rabbit monoclonal, 38 kDa; 1:1000 dilution; Abcam plc 32127). After three rinses in TBST buffer, the secondary antibody (HPR-conjugated anti-mouse; 1/1000 dilution; Dako, Glostrup, Denmark) was added, and the membranes were incubated for 1 h at room temperature. After the membranes were rinsed, protein expression levels were detected by enhanced chemiluminescence and visualized by autoradiography (ECL). Beta-tubulin (mouse monoclonal, 54 KDa; 1:4000 dilution; Sigma) was utilized as the loading control.

Statistical analysis

Statistical procedures were performed using SPSS 20.0 software (SPSS, Chicago, IL, United States). The results were reported as the mean ± SEM. Statistical analyses of the data were performed by the Student's *t*-test. Differences were set as significant at $P < 0.05$.

RESULTS

Gastric mucosal damage induced by RWIS

A macroscopic analysis showed no gastric mucosal damage in the control group, whereas scattered spotty and linear haemorrhage was observed in the gastric mucosa of rats in the RWIS group (Figure 1). The damage index was significantly different between the control (0.0 ± 0.0) and RWIS groups (47.5 ± 11.1) ($P < 0.01$).

Identification of differentially expressed proteins

Relative protein expression values were compared between the control and RWIS groups to identify the differentially expressed proteins. This analysis indentified 2853 proteins (Table S1), including 65 unique (31 significantly upregulated and 34 downregulated) proteins that were dysregulated (fold change ratio ≥ 1.2, with $P < 0.05$) (Figure 2A). These upregulated proteins (*e.g.*, cell division cycle-16, CDC16) might be involved in cell division, and the downregulated proteins (*e.g.*, GSK3B) might be related to motor neuron axon guidance, nutrient delivery to the nervous system, and neurotransmitter regulation (Table S2). Based on the premise that each dysregulated protein was assigned at least one term, GO annotation was applied to reveal the CC, MF, and BP of the identified proteins. Of all the dysregulated proteins, 18% (eight proteins) were annotated as cytoplasm-associated proteins, and the other main location categories of the proteins are the extracellular exosomes, cytoplasm, and cytosol (13%) and the nucleus, membrane, and plasma membrane (11%) (Figure 2B). The molecular functions of the dysregulated proteins are associated with poly(A) RNA binding, ATP binding, metal binding, protein homodimerization activity, and other processes (Figure 3A). GO annotation analysis showed that the 65 dysregulated proteins are mainly involved in biological processes, such as response to oxidative stress, inflammatory response, response to peptide hormones, translation, protein phosphorylation, protein oligomerization, response to glucose, and pigmentation (Figure 3B).

IPA network analysis of differentially expressed proteins

Because the molecular function of proteins in organisms is not accomplished by proteins alone but rather in coordination with many biological molecules, the roles of differentially expressed proteins were further analysed using the IPA tool according to -log (*P*-value). The IPA analysis identified 123 signalling pathways, and the highest-ranked canonical IPA pathways are shown in Table 2. Specifically, protein kinase A signalling was found to be significantly negative. The signalling pathways of the dysregulated proteins are mainly associated with cardiovascular system development and function and organ morphology, which comprise the first tier. The second tier consists of infectious diseases, digestive system development and function, gastrointestinal disease, organismal development, and organismal injury and abnormalities, and the third tier comprises cellular growth and proliferation, neurological disease, and haematological system development and function. Notably, GSK3B participates in five of the top ten most enriched pathways (Table 2) and is related to all the above-mentioned functions. Moreover, the dysregulated proteins were co-enriched into five networks, three of which are related to nerve signalling pathways (Figure 4). The highest-scoring IPA network contained 34 focus molecules and six upregulated and 10 downregulated proteins that met the significance

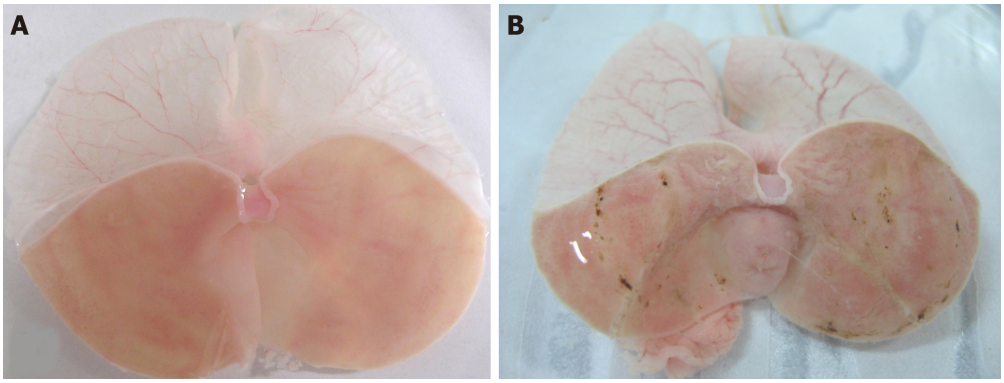


Figure 1 Gastric mucosal damage induced by restraint water-immersion stress. A: Control group. The gastric mucosal surface was intact and smooth; B: Stressed group. The gastric mucosal surface showed spotty and linear haemorrhages after 4 h of restraint water-immersion stress.

criterion, including GSK3B; these proteins are mostly involved in cardiovascular system development and function, organ morphology, and neurological disease signalling pathways (Figure 4A).

Western blot validation

According to both the GO and IPA network analyses, GSK3B was downregulated (0.71-fold change) and might affect synaptic plasticity[45]. SYN, which is involved in the process of synaptic neurotransmission, was not among the 2853 proteins showing significant expression changes between the groups. The GSK3B and SYN expression levels were validated at the protein level by Western blot, and beta-tubulin was included as a control for protein loading quantification. As shown in Figure 5, the protein level of GSK3B was significantly downregulated (n = 9, P = 0.037) in rats after RWIS for 4 h, whereas the protein level of SYN did not differ between the two groups (n = 9, P = 0.069). These results are closely consistent with the iTRAQ analysis results. Because the proteomic and WB data are quite comparable and consistent with each other, GSK3B might be related to the central mechanism of gastrointestinal dysfunction induced by RWIS, but the underlying molecular mechanisms need to be further dissected.

DISCUSSION

Many previous studies focused on the peripheral and primary central regulatory mechanisms of SGU[9-15], but little is known about the higher central mechanism[17-20,46]. This study constitutes the first analysis of the molecular differences in the MD at the proteome level between control and RWIS-exposed rats and thus, provides further insights into the molecular mechanisms and pathogenesis of SGU. Screening potential curative drugs for SGU has created an urgent need to identify new targets and iTRAQ becomes a powerful tool to explore the response in proteins to stress. Among the 2853 identified proteins, 65 were dysregulated, and these included 31 upregulated and 34 downregulated proteins. Functional analysis indentified 29 unique proteins, such as Rab31, GSK3B, and Lynx1, and these 29 proteins were primarily related to the nervous system, due to their involvement in synaptic vesicle exocytosis and endocytosis, neuron morphogenesis, and particularly neuritis, which indicates that some differences in synaptic plasticity might exist between the control and stressed groups[47-49].

To further verify the data acquired by iTRAQ proteomics, the expression of the downregulated protein GSK3B and the unchanged protein SYN, which are used as specific markers to reflect changes in the synaptic plasticity of the presynaptic terminal, was assessed by Western blot using commercially available antibodies. The Western blot results are consistent with the iTRAQ findings. The 0-3 fold change in expression obtained by Western blot analysis, which was notably greater than the changes obtained in the the proteomic analysis, might be due to the cascade amplification of optical signals during the Western blot analysis for GSK3B using the primary and secondary antibodies.

GO annotation analysis showed that the molecular functions of the 65 identified proteins are mostly associated with protein binding, including poly(A) RNA binding and ATP binding. RNA-binding proteins are dynamic posttranscriptional regulators of gene expression, whereas ATP-binding proteins are known to play a fundamental

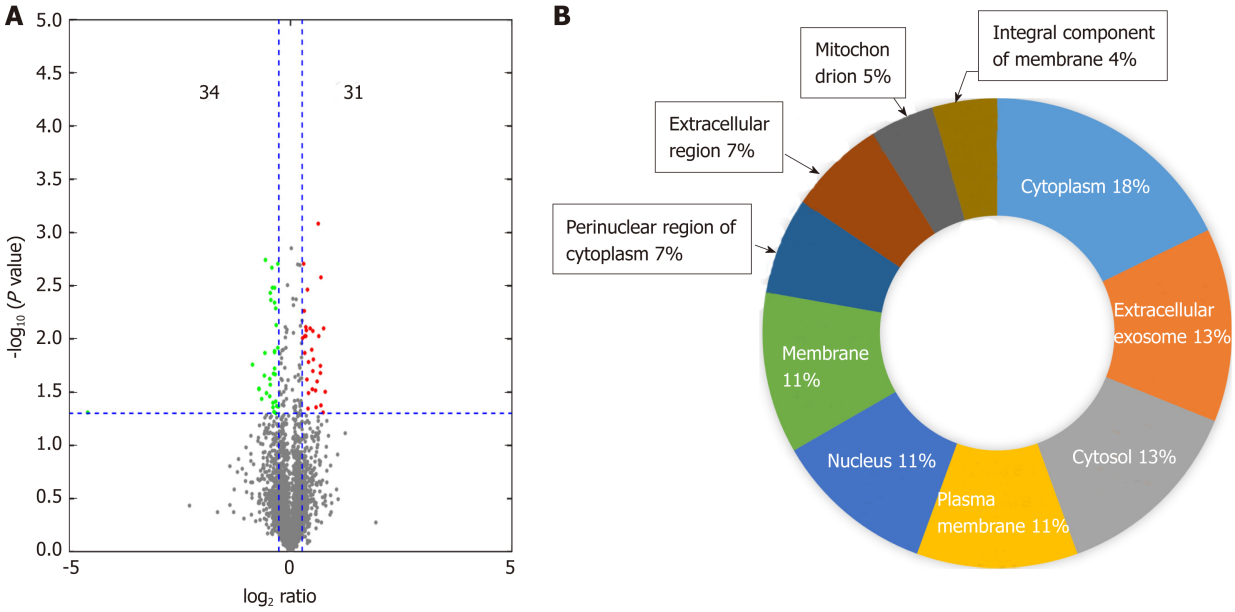


Figure 2 Features of the proteomic data from rats subjected to restraint water-immersion stress for 0 h and 4 h. The data were obtained by isobaric tags for relative and absolute quantitation shotgun analysis. A: Volcano plot indicating the changes in protein abundance in the mediodorsal thalamic nucleus between the control and stressed rats. A total of 65 dysregulated proteins with at least a ±1.2-fold change and $P < 0.05$ were identified. Orange dots indicate the upregulated proteins, and blue dots indicate the downregulated proteins; B: Subcellular localizations of the identified proteins.

role in biological processes, which indicates that synthetic and metabolic processes undergo changes in response to RWIS. The biological processes involving the dysregulated proteins were found to be associated with oxidative stress, inflammatory response, and protein phosphorylation, indicating that inflammatory disorders and abnormal metabolic functions occur in rats after RWIS for 4 h. Furthermore, some of the dysregulated proteins, such as glutathione S-transferases (GSTs), have dehydrogenase activity, which also indicates that the organism has evolved a mechanism that protects cells against reactive oxygen species during exposure to RWIS, similarly to that found in response to other stressors[50-52].

According to IPA pathway analysis, the dysregulated proteins are mainly involved in pathways related to oxidative stress, lipid and amino acid metabolism, hepatotoxicity, and kidney damage. The protein kinase A signalling pathway is the only one of the top-ranked IPA pathways that was significantly downregulated in rats after RWIS. This kinase signalling pathway plays a critical role in multiple cellular processes through the post-translational phosphorylation of various proteins, including signalling and metabolic enzymes, transcription factors, and ion channels. The altered proteins involved in this signalling pathway include two downregulated proteins, calcium/calmodulin-dependent kinase II (CAMK2D) and GSK3B, and two upregulated proteins, protein tyrosine phosphatase non-receptor type 9 (PTPN9) and cell division cycle-16 (CDC16). CAMK2, one of the most abundant protein kinases in the brain, plays a key role in neurotransmission, synaptic plasticity, and brain synapse development and showed neuroprotective activity both *in vitro* and *in vivo*[53,54]. Previous studies have found that CAMK2 can be rapidly activated and then inactivated after focal ischaemia and that the degree of inactivation is correlated with the extent of neuronal damage[55,56]. In the present study, CAMK2D was found to be downregulated in the stressed group, which indicates that the neurons in the MD were damaged during the 4-h exposure to RWIS. GSK3B is the major kinase phosphorylating tau protein and plays an important role in various physiological and pathological processes, such as glycometabolism, cell proliferation, immune response, inflammatory response, tumourigenesis, and neural development, degeneration, and regeneration[57-59]. The inhibition of GSK3 activity can promote neurite regeneration[60,61]. In this study, GSK3B was found to be downregulated, which indicates that RWIS injures neurons in the MD. However, because GSK3B can participate in many enriched pathways involved in energy metabolism, inflammation, endoplasmic reticulum stress, mitochondrial dysfunction and apoptosis pathways and so on, the molecular regulatory mechanism of GSK3 in gastric stress should be further studied. PTPN9, a cytoplasmic protein tyrosine phosphatase, is considered a marker of a tumour suppressor[62]. CDC16 was found in eukaryotic organisms and has been demonstrated to be involved in cell division[63,64]. The upregulation of PTPN9 and

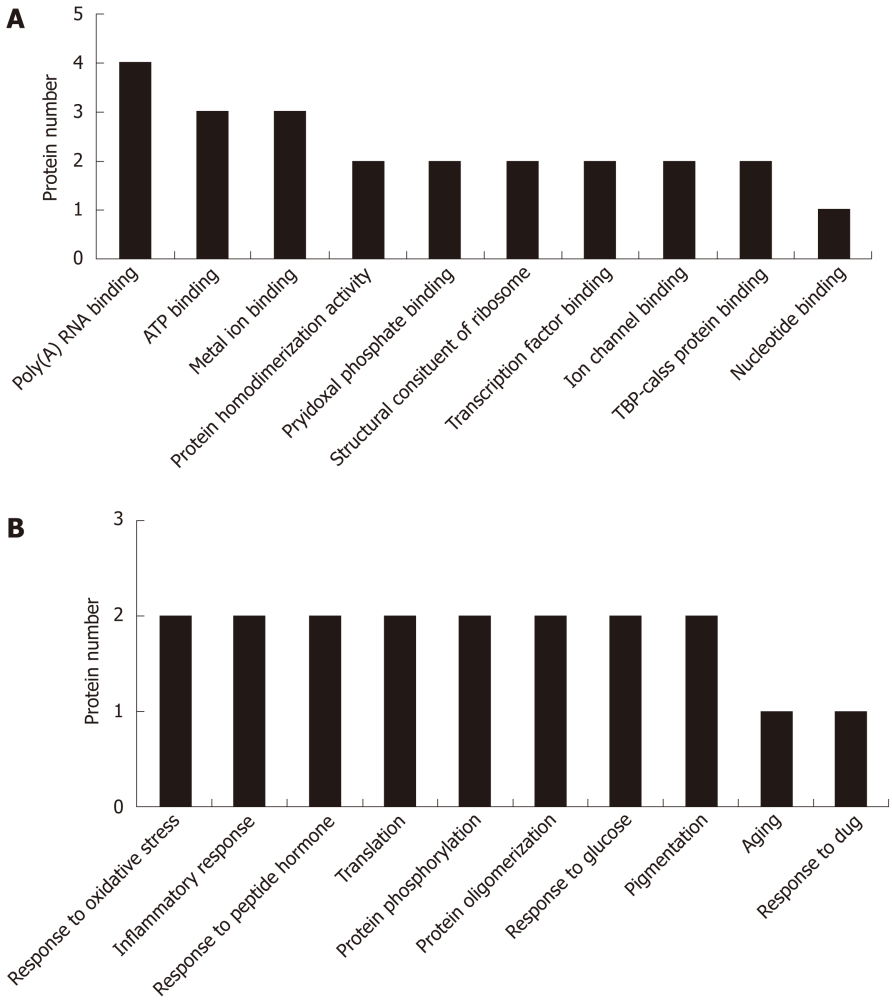


Figure 3 Features of the proteomic data from the mediodorsal thalamic nucleus of rats exposed to restraint-water-immersion-stress. The data were obtained by isobaric tags for relative and absolute quantitation shotgun analysis. A: Gene ontology (GO) analysis of the involved molecular functions; B: GO analysis of the involved biological processes. Only the top ten terms are shown.

CDC16 in rats after RWIS, as observed in the present study, will be an interesting topic that should be investigated in future studies using other methods[65].

In conclusion, an iTRAQ-based quantitative proteomic approach has been applied to detect and quantitatively analyse the alteration in the protein expression profile of the MD of rats subjected to RWIS. RWIS-induced proteomic alterations indicate that the neurons in the MD are disordered and show an abnormal function after 4 h of RWIS. Because heavy metabolic activity indicates great potential for plasticity and regeneration, the altered proteins might play important roles in cellular energy conservation and translation regulation to prevent the synthesis of unwanted proteins that could interfere with the cellular response to stress. Therefore, this study provides resources for identifying the biological functions of dysregulated proteins and dissecting pathways that could aid the identification of the molecular regulatory mechanism of SGU. The functions of key proteins linked to gastric ulcer, *e.g.*, GSK3B, during RWIS should later be verified by QRT-PCR or immunoblotting, and further functional studies using RNAi.

ACKNOWLEDGEMENTS

The mass technical service was supported by Jiyun Biotech.

Table 2 Top ten most enriched pathways indentified by an ingenuity pathway analysis of the dysregulated proteins according to –log (*P*-value)

Pathway name	-log (*P*-value)	Enriched dysregulated proteins
NRF2-mediated oxidative stress response	3.69	GSTM2, DNAJB1, GSK3B, CBR1, DNAJC11
EIF2 signalling	3.42	PTBP1, RPL18A, RPL3, EIF2S3, GSK3B
Fatty acid β-oxidation I	2.44	ACSL5, HSD17B4
Cysteine biosynthesis/homocysteine degradation	2.25	CBS/CBSL
Caveolar-mediated endocytosis signalling	1.77	ARCN1, ITGA6
Melatonin signalling	1.77	CAMK2D, ARAF
Integrin signalling	1.63	WASL, ITGA6, GSK3B
Protein kinase A signalling	1.62	CAMK2D, PTPN9, GSK3B, CDC16
Prostanoid biosynthesis	1.6	PTGDS
Reelin signalling in neurons	1.56	ITGA6, GSK3B

ACSL5: Long-chain acyl-CoA synthetase 5; ARAF: Serine/threonine-protein kinase; ARCN1: Archain 1; CAMK2D: Calcium/calmodulin-dependent kinase II; CBR1: Cannabinoid receptor 1; CBS/CBSL: Cystathionine beta-synthase; CDC16: Cell division cycle16; DNAJB1: DnaJ homolog subfamily B member I; DNAJC11: Rattus norvegicus DnaJ (Hsp40) homolog, subfamily C, member 11; EIF2S3: Eukaryotic translation initiation factor (eIF) 2 subunit 3; HSD17B4: 17β-Hydroxysteroid dehydrogenaseIV; GSK3B: Glycogen synthase kinase 3B; GSTM2: Glutathione-s-transferase μ 2; CBR1: Canine carbonyl reductase 1; ITGA6: Integrin α6; PTGDS: Prostaglandin D2 synthase; PTBP1: Polypyrimidine tract-binding protein 1; PTPN9: Protein tyrosine phosphatase non-receptor type 9; RPL3: Ribosomal protein L3; RPL18A: Ribosomal protein L18A; WASL: Wiskott aldrich syndrome like protein.

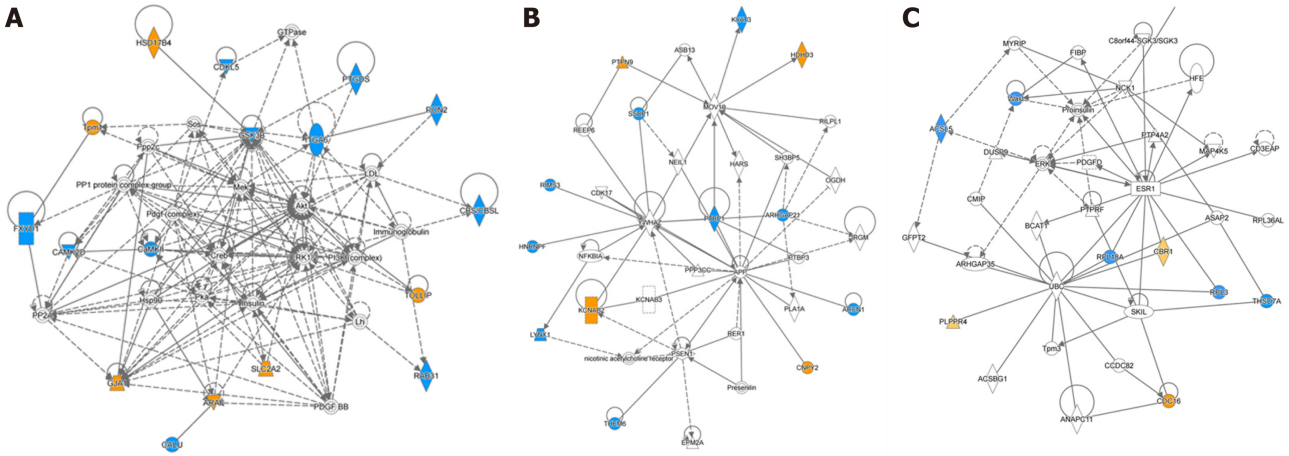


Figure 4 Key signalling pathways involving the proteins that were differentially expressed in the mediodorsal thalamic nucleu of rats exposed to restraint water-immersion stress. A: Cardiovascular system development and function, organ morphology, and neurological disease; B: Cellular assembly and organization, nervous system development and function, and tissue morphology; C: Cellular development, cellular growth and proliferation, and nervous system development and function. Orange labels indicate the upregulated proteins, and blue labels indicate the downregulated proteins.

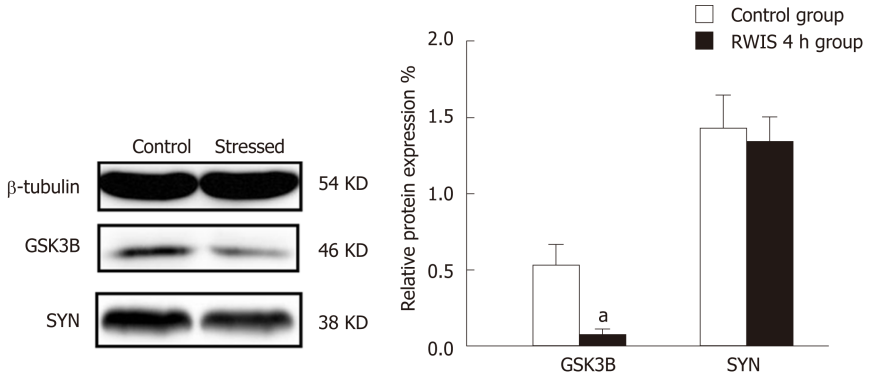


Figure 5 Verification of glycogen synthase kinase-3 beta and synaptophysin expression by Western blot. After brain dissection, the proteins in the mediodorsal thalamic nucleu were separated by SDS-PAGE, and the expression levels of glycogen synthase kinase-3 beta (GSK3B) and synaptophysin (SYN) were detected by Western blot using antibodies against GSK3B and SYN. The bars represent the changes in the total GSK3B and SYN levels. Each value is the mean ± SD from at least three independent experiments; $^{a}P < 0.05$. The expression of GSK3B was increased in the stressed group, whereas SYN showed no significant difference, which is in agreement with the isobaric tags for relative and absolute quantitation results.

ARTICLE HIGHLIGHTS

Research background

Stress-induced gastric ulcer (SGU) is one of the most common visceral complications after trauma. Restraint water-immersion stress (RWIS) can cause serious gastrointestinal dysfunction and has been widely used to study the pathogenesis of SGU to identify medications that can cure the disease. We have focused on providing a resource for determining the molecular regulatory mechanisms of stress-induced gastric mucosal lesion since 1990s. The mediodorsal thalamic nucleus (MD) is the centre integrating visceral and physical activity. There is remarkable Fos expression in the MD of rats subjected to RWIS.

Research motivation

iTRAQ becomes a powerful tool to explore the response in proteins to stress. A comparative proteomic analysis of RWIS-exposed and control rats might not only shed light on the role of the MD in gastrointestinal dysfunction induced by RWIS but also contribute to the detection of proteomic differences and the identification of targets for more specific therapies.

Recearch objectives

To screen for differentially expressed proteins in the MD of the RWIS rats to further elucidate the molecular mechanisms of SGU.

Research methods

Male Wistar rats were selected randomly and divided into two groups, namely, a control group and an RWIS group. Gastric mucosal lesions of the sacrificed rats were measured using the erosion index (EI) and the proteomic profiles of the MD were generated through isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two-dimensional liquid chromatography and tandem mass spectrometry (LC–MS/MS). Additionally, iTRAQ results were verified by Western blot analysis.

Research results

A total of 2853 proteins were identified, and these included 65 dysregulated (31 upregulated and 34 downregulated) proteins (fold change ratio ≥ 1.2). Gene Ontology (GO) analysis showed that most of the upregulated proteins are primarily related to cell division, whereas most of the downregulated proteins are related to neuron morphogenesis and neurotransmitter regulation. Ingenuity Pathway Analysis (IPA) analysis revealed that the dysregulated proteins are mainly involved in the neurological disease signalling pathways. Furthermore, our results indicated that glycogen synthase kinase-3 beta (GSK3B) might be related to the central mechanism through which RWIS gives rise to SGU.

Research conclusions

Quantitative proteomic analysis elucidates the molecular targets associated with the production of SGU and provides insights into the effects of the MD. The underlying molecular mechanisms need to be further dissected.

Research perspectives

This study provides resources for identifying the biological functions of dysregulated proteins and dissecting pathways that could aid the identification of the molecular regulatory mechanism of SGU. The functions of key proteins linked to gastric ulcer, *e.g.*, GSK3B, during RWIS should later be verified by qrt-pCR or immunoblotting, and further functional studies using RNAi.

REFERENCES

1 **Yang G**, Guo H, Li H, Shan S, Zhang X, Rombout JH, An L. Molecular characterization of LEAP-2 cDNA in common carp (Cyprinus carpio L.) and the differential expression upon a Vibrio anguillarum stimulus; indications for a significant immune role in skin. *Fish Shellfish Immunol* 2014; **37**: 22-29 [PMID: 24418455 DOI: 10.1016/j.fsi.2014.01.004]

2 **Rombout JH**, Yang G, Kiron V. Adaptive immune responses at mucosal surfaces of teleost fish. *Fish Shellfish Immunol* 2014; **40**: 634-643 [PMID: 25150451 DOI: 10.1016/j.fsi.2014.08.020]

3 **Yang HT**, Zou SS, Zhai LJ, Wang Y, Zhang FM, An LG, Yang GW. Pathogen invasion changes the intestinal microbiota composition and induces innate immune responses in the zebrafish intestine. *Fish Shellfish Immunol* 2017; **71**: 35-42 [PMID: 28964859 DOI: 10.1016/j.fsi.2017.09.075]

4 **Saxena B**, Singh S. Comparison of three acute stress models for simulating the pathophysiology of stress-related mucosal disease. *Drug Discov Ther* 2017; **11**: 98-103 [PMID: 28320982 DOI: 10.5582/ddt.2016.01081]

5 **Liu M**, Xie S, Zhou J. Use of animal models for the imaging and quantification of angiogenesis. *Exp Anim* 2018; **67**: 1-6 [PMID: 28757511 DOI: 10.1538/expanim.17-0054]

6 **Travagli RA**, Hermann GE, Browning KN, Rogers RC. Brainstem circuits regulating gastric function. *Annu Rev Physiol* 2006; **68**: 279-305 [PMID: 16460274 DOI: 10.1146/annurev.physiol.68.040504.094635]

7 **Cruz MT**, Murphy EC, Sahibzada N, Verbalis JG, Gillis RA. A reevaluation of the effects of stimulation of the dorsal motor nucleus of the vagus on gastric motility in the rat. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R291-R307 [PMID: 16990483 DOI: 10.1152/ajpregu.00863.2005]

8 **Zhou SY**, Lu YX, Yao H, Owyang C. Spatial organization of neurons in the dorsal motor nucleus of the vagus synapsing with intragastric cholinergic and nitric oxide/VIP neurons in the rat. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G1201-G1209 [PMID: 18460697 DOI: 10.1152/ajpgi.00309.2006]

9 **Li YM**, Lu GM, Zou XP, Li ZS, Peng GY, Fang DC. Dynamic functional and ultrastructural changes of gastric parietal cells induced by water immersion-restraint stress in rats. *World J Gastroenterol* 2006; **12**: 3368-3372 [PMID: 16733853 DOI: 10.3748/wjg.v12.i21.3368]

10 **Huang P**, Zhou ZR, Zheng MQ, Shi FX. Effect of the IGF-1/PTEN/Akt/FoxO signaling pathway in the duodenal mucosa of rats subjected to water immersion and restraint stress. *Genet Mol Res* 2012; **11**: 4775-4788 [PMID: 23079979 DOI: 10.4238/2012.September.19.2]

11 **Huang P**, Zhou Z, Wang H, Wei Q, Zhang L, Zhou X, Hutz RJ, Shi F. Effect of the IGF-1/PTEN/Akt/FoxO signaling pathway on the development and healing of water immersion and restraint stress-induced gastric ulcers in rats. *Int J Mol Med* 2012; **30**: 650-658 [PMID: 22735908 DOI: 10.3892/ijmm.2012.1041]

12 **Zhou ZR**, Huang P, Song GH, Zhang Z, An K, Lu HW, Ju XL, Ding W. Comparative proteomic analysis of rats subjected to water immersion and restraint stress as an insight into gastric ulcers. *Mol Med Rep* 2017; **16**: 5425-5433 [PMID: 28849061 DOI: 10.3892/mmr.2017.7241]

13 **Zhang YY**, Cao GH, Zhu WX, Cui XY, Ai HB. Comparative study of c-Fos expression in rat dorsal vagal complex and nucleus ambiguus induced by different durations of restraint water-immersion stress. *Chin J Physiol* 2009; **52**: 143-150 [PMID: 19777800]

14 **Zhang YY**, Zhu WX, Cao GH, Cui XY, Ai HB. c-Fos expression in the supraoptic nucleus is the most intense during different durations of restraint water-immersion stress in the rat. *J Physiol Sci* 2009; **59**: 367-375 [PMID: 19484338 DOI: 10.1007/s12576-009-0044-8]

15 **Wang H**, Liu WJ, Shen GM, Zhang MT, Huang S, He Y. Neural mechanism of gastric motility regulation by electroacupuncture at RN12 and BL21: A paraventricular hypothalamic nucleus-dorsal vagal complex-vagus nerve-gastric channel pathway. *World J Gastroenterol* 2015; **21**: 13480-13489 [PMID: 26730159 DOI: 10.3748/wjg.v21.i48.13480]

16 **Zhao DQ**, Lu CL, Ai HB. The role of catecholaminergic neurons in the hypothalamus and medullary visceral zone in response to restraint water-immersion stress in rats. *J Physiol Sci* 2011; **61**: 37-45 [PMID: 21161464 DOI: 10.1007/s12576-010-0119-6]

17 **Zhao DQ**, Ai HB. Oxytocin and vasopressin involved in restraint water-immersion stress mediated by oxytocin receptor and vasopressin 1b receptor in rat brain. *PLoS One* 2011; **6**: e23362 [PMID: 21858088 DOI: 10.1371/journal.pone.0023362]

18 **Zhu J**, Chang L, Xie J, Ai H. Arginine Vasopressin Injected into the Dorsal Motor Nucleus of the Vagus Inhibits Gastric Motility in Rats. *Gastroenterol Res Pract* 2016; **2016**: 4618672 [PMID: 26843857 DOI: 10.1155/2016/4618672]

19 **Zhao DQ**, Ai HB. Influence of restraint water-immersion stress on activities of cholinergic and catecholaminergic neurons in the nucleus ambiguous in rats. *J Shandong University (Health Science)* 2011; **49**: 103-106

20 **Sun HZ**, Zheng S, Lu K, Hou FT, Bi JX, Liu XL, Wang SS. Hydrogen sulfide attenuates gastric mucosal injury induced by restraint water-immersion stress via activation of K ATP channel and NF-κB dependent pathway. *World J Gastroenterol* 2017; **23**: 87-92 [PMID: 28104983 DOI: 10.3748/wjg.v23.i1.87]

21 **Velayos JL**, Reinoso-Suarez F. Topographic organization of the brainstem afferents to the mediodorsal thalamic nucleus. *J Comp Neurol* 1982; **206**: 17-27 [PMID: 7096629 DOI: 10.1002/cne.902060104]

22 **Groenewegen HJ**. Organization of the afferent connections of the mediodorsal thalamic nucleus in the rat, related to the mediodorsal-prefrontal topography. *Neuroscience* 1988; **24**: 379-431 [PMID: 2452377]

23 **Gritti I**, Mariotti M, Mancia M. Limbic and brainstem afferents to thalamic mediodorsal nucleus: a horseradish peroxidase study. *Neurosci Lett* 1987; **76**: 345-350 [PMID: 3587765]

24 **Ito HT**, Zhang SJ, Witter MP, Moser EI, Moser MB. A prefrontal-thalamo-hippocampal circuit for goal-directed spatial navigation. *Nature* 2015; **522**: 50-55 [PMID: 26017312 DOI: 10.1038/nature14396]

25 **Alcaraz F**, Marchand AR, Courtand G, Coutureau E, Wolff M. Parallel inputs from the mediodorsal thalamus to the prefrontal cortex in the rat. *Eur J Neurosci* 2016; **44**: 1972-1986 [PMID: 27319754 DOI: 10.1111/ejn.13316]

26 **Kuramoto E**, Pan S, Furuta T, Tanaka YR, Iwai H, Yamanaka A, Ohno S, Kaneko T, Goto T, Hioki H. Individual mediodorsal thalamic neurons project to multiple areas of the rat prefrontal cortex: A single neuron-tracing study using virus vectors. *J Comp Neurol* 2017; **525**: 166-185 [PMID: 27275581 DOI: 10.1002/cne.24054]

27 **Ruggiero DA**, Anwar S, Kim J, Glickstein SB. Visceral afferent pathways to the thalamus and olfactory tubercle: behavioral implications. *Brain Res* 1998; **799**: 159-171 [PMID: 9666113]

28 **Xu W**, Südhof TC. A neural circuit for memory specificity and generalization. *Science* 2013; **339**: 1290-1295 [PMID: 23493706 DOI: 10.1126/science.1229534]

29 **Mitchell AS**, Chakraborty S. What does the mediodorsal thalamus do? *Front Syst Neurosci* 2013; **7**: 37 [PMID: 23950738 DOI: 10.3389/fnsys.2013.00037]
30 **Mitchell AS**, Sherman SM, Sommer MA, Mair RG, Vertes RP, Chudasama Y. Advances in understanding mechanisms of thalamic relays in cognition and behavior. *J Neurosci* 2014; **34**: 15340-15346 [PMID: 25392501 DOI: 10.1523/JNEUROSCI.3289-14.2014]
31 **Mitchell AS**. The mediodorsal thalamus as a higher order thalamic relay nucleus important for learning and decision-making. *Neurosci Biobehav Rev* 2015; **54**: 76-88 [PMID: 25757689 DOI: 10.1016/j.neubiorev.2015.03.001]
32 **Wolff M**, Alcaraz F, Marchand AR, Coutureau E. Functional heterogeneity of the limbic thalamus: From hippocampal to cortical functions. *Neurosci Biobehav Rev* 2015; **54**: 120-130 [PMID: 25446945 DOI: 10.1016/j.neubiorev.2014.11.011]
33 **Wimmer RD**, Schmitt LI, Davidson TJ, Nakajima M, Deisseroth K, Halassa MM. Thalamic control of sensory selection in divided attention. *Nature* 2015; **526**: 705-709 [PMID: 26503050 DOI: 10.1038/nature15398]
34 **Browning PG**, Chakraborty S, Mitchell AS. Evidence for Mediodorsal Thalamus and Prefrontal Cortex Interactions during Cognition in Macaques. *Cereb Cortex* 2015; **25**: 4519-4534 [PMID: 25979086 DOI: 10.1093/cercor/bhv093]
35 **Wolff M**, Faugère A, Desfosses É, Coutureau É, Marchand AR. Mediodorsal but not anterior thalamic nuclei lesions impair acquisition of a conditional discrimination task. *Neurobiol Learn Mem* 2015; **125**: 80-84 [PMID: 26254715 DOI: 10.1016/j.nlm.2015.07.018]
36 **Sherman SM**. Thalamus plays a central role in ongoing cortical functioning. *Nat Neurosci* 2016; **19**: 533-541 [PMID: 27021938 DOI: 10.1038/nn.4269]
37 **Herrera DG**, Robertson HA. Activation of c-fos in the brain. *Prog Neuro Bio* 1996; **l50**: 83-107 [DOI: 10.1016/S0301-0082(96)00021-4]
38 **Zhang F**, Wang XJ, Huang YH, Zhao ZG, Zhang SS, Gong XS, Xie L, Kang DM, Jing X. Differential expression of hemolymph proteins between susceptible and insecticide-resistant Blattella germanica (Blattodea: Blattellidae). *Environ Entomol* 2014; **43**: 1117-1123 [PMID: 25182623 DOI: 10.1603/EN13351]
39 **Lessner G**, Schmitt O, Haas SJ, Mikkat S, Kreutzer M, Wree A, Glocker MO. Differential proteome of the striatum from hemiparkinsonian rats displays vivid structural remodeling processes. *J Proteome Res* 2010; **9**: 4671-4687 [PMID: 20666516 DOI: 10.1021/pr100389u]
40 **Yin X**, Zhang Y, Guo S, Jin H, Wang W, Yang P. Large scale systematic proteomic quantification from non-metastatic to metastatic colorectal cancer. *Sci Rep* 2015; **5**: 12120 [PMID: 26175278 DOI: 10.1038/srep12120]
41 **Wang Q**, Su X, Jiang X, Dong X, Fan Y, Zhang J, Yu C, Gao W, Shi S, Jiang J, Jiang W, Wei T. iTRAQ technology-based identification of human peripheral serum proteins associated with depression. *Neuroscience* 2016; **330**: 291-325 [PMID: 27268281 DOI: 10.1016/j.neuroscience.2016.05.055]
42 **Guth PH**, Aures D, Paulsen G. Topical aspirin plus HCl gastric lesions in the rat. Cytoprotective effect of prostaglandin, cimetidine, and probanthine. *Gastroenterology* 1979; **76**: 88-93 [PMID: 361495]
43 **Paxinos G**, Watson C. The rat brain in stereotaxic coordinates (6th edition). Elsevier: Amsterdam 2007, 126-165.
44 **Yang CL**, Zhu HY, Zhang F. Comparative Proteomics Analysis Between the Short-Term Stress and Long-Term Adaptation of the Blattella germanica (Blattodea: Blattellidae) in Response to Beta-Cypermethrin. *J Econ Entomol* 2019 [PMID: 30835785]
45 **Sharma S**, Taliyan R. Neuroprotective role of Indirubin-3'-monoxime, a GSKβ inhibitor in high fat diet induced cognitive impairment in mice. *Biochem Biophys Res Commun* 2014; **452**: 1009-1015 [PMID: 25234596 DOI: 10.1016/j.bbrc.2014.09.034]
46 **Sun H**, Liu Z, Ma X. Interactions between astrocytes and neurons in the brainstem involved in restraint water immersion stress-induced gastric mucosal damage. *Neuroreport* 2016; **27**: 151-159 [PMID: 26720891 DOI: 10.1097/WNR.0000000000000515]
47 **Li H**, Yang G, Ma F, Li T, Yang H, Rombout JH, An L. Molecular characterization of a fish-specific toll-like receptor 22 (TLR22) gene from common carp (Cyprinus carpio L.): Evolutionary relationship and induced expression upon immune stimulants. *Fish Shellfish Immunol* 2017; **63**: 74-86 [PMID: 28192255 DOI: 10.1016/j.fsi.2017.02.009]
48 **Marsden WN**. Synaptic plasticity in depression: molecular, cellular and functional correlates. *Prog Neuropsychopharmacol Biol Psychiatry* 2013; **43**: 168-184 [PMID: 23268191 DOI: 10.1016/j.pnpbp.2012.12.012]
49 **Higley MJ**, Strittmatter SM. Neuroscience. Lynx for braking plasticity. *Science* 2010; **330**: 1189-1190 [PMID: 21109660 DOI: 10.1126/science.1198983]
50 **Sies H**, Berndt C, Jones DP. Oxidative Stress. *Annu Rev Biochem* 2017; **86**: 715-748 [PMID: 28441057 DOI: 10.1146/annurev-biochem-061516-045037]
51 **Siritantikorn A**, Johansson K, Ahlen K, Rinaldi R, Suthiphongchai T, Wilairat P, Morgenstern R. Protection of cells from oxidative stress by microsomal glutathione transferase 1. *Biochem Biophys Res Commun* 2007; **355**: 592-596 [PMID: 17306223 DOI: 10.1016/j.bbrc.2007.02.018]
52 **Sharma R**, Yang Y, Sharma A, Awasthi S, Awasthi YC. Antioxidant role of glutathione S-transferases: protection against oxidant toxicity and regulation of stress-mediated apoptosis. *Antioxid Redox Signal* 2004; **6**: 289-300 [PMID: 15025930 DOI: 10.1089/152308604322899350]
53 **Ashpole NM**, Hudmon A. Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. *Mol Cell Neurosci* 2011; **46**: 720-730 [PMID: 21316454 DOI: 10.1016/j.mcn.2011.02.003]
54 **Vest RS**, O'Leary H, Coultrap SJ, Kindy MS, Bayer KU. Effective post-insult neuroprotection by a novel Ca(2+)/ calmodulin-dependent protein kinase II (CaMKII) inhibitor. *J Biol Chem* 2010; **285**: 20675-20682 [PMID: 20424167 DOI: 10.1074/jbc.M109.088617]
55 **Hanson SK**, Grotta JC, Waxham MN, Aronowski J, Ostrow P. Calcium/calmodulin-dependent protein kinase II activity in focal ischemia with reperfusion in rats. *Stroke* 1994; **25**: 466-473 [PMID: 8303758]
56 **Zalewska T**, Domanska-Janik K. Brain ischaemia transiently activates Ca2+/calmodulin-independent protein kinase II. *Neuroreport* 1996; **7**: 637-641 [PMID: 8730847]
57 **Jope RS**, Yuskaitis CJ, Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res* 2007; **32**: 577-595 [PMID: 16944320 DOI: 10.1007/s11064-006-9128-5]
58 **Preece P**, Virley DJ, Costandi M, Coombes R, Moss SJ, Mudge AW, Jazin E, Cairns NJ. Beta-secretase (BACE) and GSK-3 mRNA levels in Alzheimer's disease. *Brain Res Mol Brain Res* 2003; **116**: 155-158 [PMID: 12941471]

59 **Kaytor MD**, Orr HT. The GSK3 beta signaling cascade and neurodegenerative disease. *Curr Opin Neurobiol* 2002; **12**: 275-278 [PMID: 12049933]

60 **Dill J**, Wang H, Zhou F, Li S. Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. *J Neurosci* 2008; **28**: 8914-8928 [PMID: 18768685 DOI: 10.1523/JNEUROSCI.1178-08.2008]

61 **Kim YT**, Hur EM, Snider WD, Zhou FQ. Role of GSK3 Signaling in Neuronal Morphogenesis. *Front Mol Neurosci* 2011; **4**: 48 [PMID: 22131966 DOI: 10.3389/fnmol.2011.00048]

62 **Hu B**, Yan X, Liu F, Zhu C, Zhou H, Chen Y, Liu J, Gu X, Ni R, Zhang T. Downregulated Expression of PTPN9 Contributes to Human Hepatocellular Carcinoma Growth and Progression. *Pathol Oncol Res* 2016; **22**: 555-565 [PMID: 26715439 DOI: 10.1007/s12253-015-0038-1]

63 **Kittler R**, Putz G, Pelletier L, Poser I, Heninger AK, Drechsel D, Fischer S, Konstantinova I, Habermann B, Grabner H, Yaspo ML, Himmelbauer H, Korn B, Neugebauer K, Pisabarro MT, Buchholz F. An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* 2004; **432**: 1036-1040 [PMID: 15616564 DOI: 10.1038/nature03159]

64 **Meng XQ**, Dai YY, Jing LD, Bai J, Liu SZ, Zheng KG, Pan J. Subcellular localization of proline-rich tyrosine kinase 2 during oocyte fertilization and early-embryo development in mice. *J Reprod Dev* 2016; **62**: 351-358 [PMID: 27086609 DOI: 10.1262/jrd.2016-015]

65 **Shan SJ**, Liu DZ, Wang L, Zhu YY, Zhang FM, Li T, An LG, Yang GW. Identification and expression analysis of irak1 gene in common carp Cyprinus carpio L.: indications for a role of antibacterial and antiviral immunity. *J Fish Biol* 2015; **87**: 241-255 [PMID: 26099328 DOI: 10.1111/jfb.12714]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2924-2934

DOI: 10.3748/wjg.v25.i23.2924

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Effects of *Bifidobacterium infantis* on cytokine-induced neutrophil chemoattractant and insulin-like growth factor-1 in the ileum of rats with endotoxin injury

Wei Wang, Mei Sun, Yu-Ling Zheng, Liu-Yu Sun, Shu-Qiang Qu

ORCID number: Wei Wang (0000-0001-8512-5395); Mei Sun (0000-0003-1497-6912); Yu-Ling Zheng (0000-0001-7522-8091); Liu-Yu Sun (0000-0002-8050-4772); Shu-Qiang Qu (0000-0002-3604-6524).

Author contributions: Wang W and Sun M designed the experiment; all authors took part in this experiment; Zheng Y collected the data; Sun L performed analysis of the data; all authors participated in writing and critical revision of the manuscript; Qu S provided final approval of the manuscript.

Supported by the Education Department of Heilongjiang Province, China, No. 11521124.

Institutional review board statement: This study was approved by the Institutional Review Board of the Second Affiliated Hospital of Harbin Medical University.

Institutional animal care and use committee statement: This study was approved by the Institutional Animal Ethical Review Board of the Second Affiliated Hospital of Harbin Medical University (SYDW2018-115).

Conflict-of-interest statement: No conflict of interest exists.

Data sharing statement: No additional data sharing.

ARRIVE guidelines statement: The ARRIVE Guidelines have been adopted.

Wei Wang, Yu-Ling Zheng, Liu-Yu Sun, Shu-Qiang Qu, Department of Pediatrics, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China

Mei Sun, Department of Pediatric Gastroenterology, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

Corresponding author: Shu-Qiang Qu, PhD, Chief Doctor, Director, Professor, Teacher, Pediatrician, Department of Pediatrics, The Second Affiliated Hospital of Harbin Medical University, No. 246 Xuefu Road, Harbin 150086, Heilongjiang Province, China. qushuqiang1962@hotmail.com
Telephone: +86-451-86297730
Fax: +86-451-86297730

Abstract

BACKGROUND

The digestive tract is the maximal immunizing tissue in the body, and mucosal integrity and functional status of the gut is very important to maintain a healthy organism. Severe infection is one of the most common causes of gastrointestinal dysfunction, and the pathogenesis is closely related to endotoxemia and intestinal barrier injury. *Bifidobacterium* is one of the main probiotics in the human body that is involved in digestion, absorption, metabolism, nutrition, and immunity. *Bifidobacterium* plays an important role in maintaining the intestinal mucosal barrier integrity. This study investigated the protective mechanism of *Bifidobacterium* during ileal injury in rats.

AIM

To investigate the effects of *Bifidobacterium* on cytokine-induced neutrophil chemoattractant (CINC) and insulin-like growth factor 1 (IGF-1) in the ileum of rats with endotoxin injury.

METHODS

Preweaning rats were randomly divided into three groups: Control (group C), model (group E) and treatment (group T). Group E was intraperitoneally injected with lipopolysaccharide (LPS) to create an animal model of intestinal injury. Group T was intragastrically administered *Bifidobacterium* suspension 7 d before LPS. Group C was intraperitoneally injected with normal saline. The rats were killed at 2, 6 or 12 h after LPS or physiological saline injection to collect ileal

Manuscript source: Unsolicited manuscript

Received: November 21, 2018
Peer-review started: November 22, 2018
First decision: December 12, 2018
Revised: March 12, 2019
Accepted: March 29, 2019
Article in press: March 30, 2019
Published online: June 21, 2019

P-Reviewer: Higuchi K, Jung DH, Pourshafie MR
S-Editor: Yan JP
L-Editor: Filipodia
E-Editor: Ma YJ

tissue samples. The expression of ileal CINC mRNA was evaluated by reverse transcription-polymerase chain reaction (RT-PCR), and expression of ileal IGF-1 protein and mRNA was detected by immunohistochemistry and RT-PCR, respectively.

RESULTS

The ileum of rats in Group C did not express CINC mRNA, ileums from Group E expressed high levels, which was then significantly decreased in Group T (F = 23.947, P < 0.05). There was no significant difference in CINC mRNA expression at different times (F = 0.665, P > 0.05). There was a high level of IGF-1 brown granules in ileal crypts and epithelial cells in Group C, sparse staining in Group E, and dark, dense brown staining in Group T. There was a significant difference between Groups C and E and Groups E and T (P < 0.05). There was no significant difference in IGF-1 protein expression at different times (F = 1.269, P > 0.05). IGF-1 mRNA expression was significantly different among the three groups (P < 0.05), though not at different times (F = 0.086, P > 0.05).

CONCLUSION

Expression of CINC mRNA increased in the ileum of preweaning rats with endotoxin injury, and exogenous administration of *Bifidobacterium* reduced CINC mRNA expression. IGF-1 protein and mRNA expression decreased in the ileum of preweaning rats with endotoxin injury, and exogenous administration of *Bifidobacterium* prevented the decrease in IGF-1 expression. *Bifidobacterium* may increase IGF-1 expression and enhance intestinal immune barrier function in rats with endotoxin injury.

Key words: *Bifidobacterium*; Ileum; Cytokine-induced neutrophil chemoattractant; Insulin-like growth factor-1; Rats

Core tip: The purpose of this article is to investigate the effects of *Bifidobacterium* on cytokine-induced neutrophil chemoattractant and insulin-like growth factor 1 (IGF-1) in the ileum of rats with endotoxin injury. It was found that *Bifidobacterium* may increase IGF-1 expression and enhance intestinal immune barrier function in rats with endotoxin injury.

Citation: Wang W, Sun M, Zheng YL, Sun LY, Qu SQ. Effects of *Bifidobacterium infantis* on cytokine-induced neutrophil chemoattractant and insulin-like growth factor-1 in the ileum of rats with endotoxin injury. *World J Gastroenterol* 2019; 25(23): 2924-2934
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2924.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2924

INTRODUCTION

Severe infection is one of the most common causes of gastrointestinal dysfunction, and the pathogenesis is closely related to endotoxemia and intestinal barrier injury. *Bifidobacterium* is one of the main probiotics in the human body involved in digestion, absorption, metabolism, nutrition, and immunity to infection. In particular, *Bifidobacterium* plays an important role in maintaining intestinal mucosal barrier integrity[1]. Insulin-like growth factor 1 (IGF-1) is a polypeptide that is primarily secreted from the liver, but it is also expressed in other organs such as the intestine, making it part of the intestinal immune barrier[2]. This study investigated the protective mechanism of *Bifidobacterium* through ileal injury in preweaning rats.

MATERIALS AND METHODS

Materials

Bifidobacterium infantis (KLDS2.0002) suspension was purchased from the Key

Laboratory of Dairy Science of Northeast Agricultural University. *Escherichia coli* (O55: B5) lipopolysaccharide (LPS) was purchased from Sigma. Immunohistochemical IGF-1 rabbit anti-rat IgG (primary antibody) was purchased from Wuhan Boshide Bioengineering Co. Ltd. (Wuhan, China), and horseradish-peroxidase-labeled IgG (secondary antibody) and concentrated DAB kits were purchased from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd (Beijing, China). The primers for IGF-1, cytokine-induced neutrophil chemoattractant (CINC) and β-actin were designed and synthesized by Shanghai Shengneng Bocai Biological Technology Co. Ltd. A reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from Promega (Beijing, China).

Instruments

Instruments and equipment were obtained as follows: ultramicrotome (LKB, Sweden); GIS gel image processing system (Tanon, Shanghai, China); TC-XP gene amplification instrument (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China); GE-100 gel electrophoresis system (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China); Eclipse E800 Camera system (Nikon, Japan); CMIAS2001 Beihang Motic Image Analysis system (Beijing Mike Audi Image Technology Co. Ltd., Beijing, China); HFsafe 1200 biosafety cabinet (LiShen Scientific Instrument Co. Ltd., Shanghai, China); AE200s electronic analytical balance (Mettler Toledo Instruments Shanghai Co. Ltd., Shanghai, China); MDF-U53V 80 °C low temperature freezer (SANSY, Japan); Neofuge 23R desktop high speed refrigerated centrifuge (Shanghai, China); microsample feeder (Eppendorf AG, Germany); and XW-80A vortex mixer (Shanghai, China).

Animals

Healthy 18-day-old preweaning Wistar rats (31.16 ± 6.38 g) were provided by the Laboratory Animal Center of the Second Affiliated Hospital of Harbin Medical University. The rats were randomly divided into three groups: Control (group C), model (group E) and *Bifidobacterium* treatment (group T). The animals were sacrificed 2, 6 and 12 h after intraperitoneal injection of endotoxin (LPS) or normal saline, and ileal samples were collected. Each group had eight preweaning rats for each time point. Animals that died during the experiment were not included.

Bifidobacterium culture

TPY medium: 10 g casein peptone, 2.5 g yeast extract powder, 5 g glucose, 5 g soy peptone, 1.0 g Tween 80, 0.5 g L-cysteine hydrochloric acid, 5 mL salt-mixture solution, and 995 mL distilled water, adjusted to pH 7.2, and sterilized at 121 °C for 20 min. Salt-mixture solution: 20.0 g K_2HPO_4, 5.0 g $MgCl_2{\cdot}6H_2O$, 2.5 g $ZnSO_4{\cdot}7H_2O$, 1.5 g $CaCl_2$ and 0.5 g $FeC1_3$, added to 100 mL distilled water.

Activation of freeze-dried strains: Before activation, cryopreserved strains were placed at room temperature for several hours. The ampoule was wiped with 70% alcohol in a sterile room, opened, and 0.2 mL TPY medium was added. The strains taken by sterilized platinum earrings were inoculated into TPY medium and onto agar plates. After anaerobic incubation for 48-72 h at 37 °C, three generations were continuously activated to enhance the viability of the strains.

Morphological observation: Platinum earrings were used to remove bacteria from a single colony or liquid culture and then smeared onto a microscope slide for Gram staining. The bacterial morphology was observed under the microscope. Generation of strains: After microscopic examination, strains with good morphology and no bacterial colonies in the solid medium were selected, and lines were drawn on TPY solid medium. At the same time, TPY liquid medium was added to bacteria to culture anaerobically at 37 °C for 48-72 h.

Measurement of viable bacteria: The 0.5 mL suspension was gradiently diluted 10 times with sterilized normal saline. The 0.2 mL suspension of the appropriate dilution gradient was extracted and placed on the TPY agar plate two or three times for each dilution gradient, and the suspension was uniformly spread out using a sterilized rod. After ventilation in the corridor of the anaerobic incubator, the suspension was transferred to an incubator for anaerobic cultivation at 37 °C for 48 h. Finally, bacterial colonies were counted after the strains were grown.

Preparation of animal models

Rats in groups E and T were intraperitoneally injected with 5 mg/kg LPS (5 mg/mL dissolved in normal saline). In group T, 0.5 mL *Bifidobacterium* suspension (2.0×10^9 CFU/mL) was administered intragastrically twice daily 1 wk before LPS, until the end of the experiment. In group C, 1 mL/kg normal saline was intraperitoneally

injected. The preweaning rats in each group were returned to their cage after treatment and continued to receive rat's milk.

Specimen collection

After the animals were sacrificed, the abdomen was aseptically dissected, and the contents of the intestinal cavity were washed with ice-cold physiological saline. A 0.5–1 cm length of ileum 2–3 cm away from the ileocecal junction was removed and fixed in 4% paraformaldehyde dissolved in 0.1 M PBS. The tissue was embedded in paraffin and sectioned for immunohistochemical study. A 4-5 cm length of ileum 3-4 cm away from the ileocecal junction was removed and placed in an RNase-free Eppendorf tube and stored at – 80 °C prior to total RNA extraction and RT-PCR.

Immunohistochemical detection of IGF-1 protein in ileum

The SP method (peroxidase-labeled streptomycin, and streptavidin/peroxidase) was used for immunohistochemistry. Paraffin sections were dewaxed and washed twice with 0.01 M PBS at pH 7.4 for 5 min. The sections were incubated in recently prepared 3% H_2O_2 (in 80% methanol) at room temperature for 5-10 min to eliminate endogenous peroxidase activity and washed three times in PBS for 5 min. Antigen retrieval was performed with 1 mM EDTA (pH 9.0) under high temperature and high pressure for 10 min. When returning to room temperature, sections were washed with distilled water, then washed three times with PBS for 5 min, covered with 10% normal goat serum (PBS diluted) and incubated at room temperature for 20 min to reduce non-specific staining. Excess liquid was discarded. A total of 50 μL rabbit anti-rat IGF-1 antibody (1:100) was incubated at 4 °C overnight or 37 °C for 1 hour, followed by three washes in PBS for 5 min each. Then, 50 μL biotin-labeled secondary antibody was incubated at 37 °C for 10-30 min, and washed three times with PBS for 5 min. Finally, 50 μL horseradish-peroxidase-labeled streptavidin was incubated at room temperature for 20 min and washed three times in PBS for 5 min. The sections were stained with newly prepared diaminobenzidine color developing agent at room temperature for 5-10 min, with the degree of dying being monitored under microscope, then washed with distilled water for 5 min. The sections were stained again with hematoxylin for 2 min, and washed with distilled water for 5 min. They were differentiated by 0.1% hydrochloric acid alcohol, and washed in flowing water for 30 min. The stained sections were dehydrated in graded series of ethanol, cleared, and sealed with gum. For the negative controls, PBS was used in place of the rabbit anti-rat IGF-1 primary antibody. The other steps were the same as above, excluding non-specific staining.

Homogeneous brown staining was considered positive. For immunohistochemical analysis, five to eight stained sections were selected for each time point. Five non-overlapping views were randomly selected under light microscopy (× 400), and the optical density of each view was measured by Nikon Eclipse E800 Image Acquisition System and Beihang Motic Pathological Image Analysis System.

RT-PCR for detection of IGF-1 and CINC mRNA in ileum

For total RNA extraction, ileal tissues preserved at –80 °C were ground in liquid nitrogen under aseptic conditions, then poured into an Eppendorf tube. TRIzol Reagent (50-200 mg tissue/mL TRIzol) was added to the ileal tissues for 5 min at room temperature to facilitate dissociation of nucleic acid–protein complexes. Protein and DNA were removed by addition of 0.2 mL chloroform, and the supernatant was extracted by rapid vibration and centrifugation at 12000 rpm at 4 °C for 15 min.

RNA was precipitated by addition of 0.5 mL isopropanol. After mixing and incubating at room temperature for 10 min, the samples were centrifuged at 12000 rpm at 4 °C for 15 min. The supernatant was discarded, and the precipitate was resuspended in 1 mL 75% ethanol (prepared in 0.1% DEPC water) and centrifuged at 7500 rpm at 4 °C for 5 min. The supernatant was discarded and dried for 5-10 min. The RNA was dissolved in RNase-free DEPC-treated water to a volume of 20 mL, mixed, and stored at – 20 °C.

For reverse transcription into cDNA, 2 μL RNA was added to the total reaction of 20 μL. The reverse transcription reaction solution was prepared as follows: 2 μL 10 × buffer, 4 μL $MgCl_2$ (25 mM), 2 μL dNTP (10 mM), 0.5 μL RNase, 0.7 μL AMV (22 U/μL), 1 μL oligo-dT 15 (50 μM), 2 μL total RNA and 7.8 μL enzyme-free water. The reverse transcription reaction was carried out at 42 °C for 15 min, 95 °C for 15 min, and 0-5 °C for 5 min. The cDNA concentration was determined by ultraviolet spectrophotometry.

Amplification of CINC, IGF-1 and β-actin was as follows: CINC, primer sequence: 5′-TGA GCT GCG CAG TCA GTG CCT GCA-3′, antisense primer sequence: 5′-ACA CCC TTT AGC ATC TTT TGG ACA-3′. The length of product was 390 bp. IGF-1, primer sequence: 5′-GCT GCC ACT TGG ATC GCT ATT C-3′; antisense primer

sequence: 5′-CGT CCC GGG TCG TTT ACA CA-3′. The length of product was 300 bp. β-actin, primer sequence: 5′-CAT CTG CTG GAA GGT GGA CA-3′; antisense primer sequence: 5′-GAG AGG GAA ATC GTG CGT GAC-3′. The length of product was 452 bp.

The total reaction system volume was 25 μL, with 2 μg cDNA (the volume of cDNA was calculated according to the cDNA concentration of each sample); each reaction system was brought to 25 μL with enzyme-free water. Other specific components and volumes were as follows: 1.0 μL $MgCl_2$ (50 mM), 2.5 μL 10 × buffer, 0.5 μL dNTPs (10 mM), and 0.5 μL *Taq* polymerase (5 U/μL). The amplification conditions for CINC were: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; then 72 °C for 7 min. The amplification conditions for IGF-1 were: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; then 72 °C for 7 min. The amplification conditions for β-actin were: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 40 s and 72 °C for 22 s; then 72 °C for 7 min.

For semi-quantitative analysis of PCR products, PCR reaction products (3-5 μL) were mixed with ethidium bromide and subjected to 2% agarose gel electrophoresis. The PCR products were quantified by ImageJ analysis, and β-actin was the internal reference for RNA detection. The relative expression of genes = optical density of tested genes/intrinsic optical density.

Statistical analysis

Statistical analyses were performed using SPSS version 21.0 software. The results were expressed as mean ± SD. The variance analysis of factorial design was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

CINC mRNA expression in ileal tissue

CINC mRNA was not expressed in group C at any time examined. There was a significant difference between groups T and E ($F = 23.947$, $P < 0.05$). There was no significant difference in expression within a group at different time points ($F = 0.665$, $P > 0.05$) (Figures 1 and 2).

IGF-1 protein expression in ileal tissue

There was some IGF-1 expression in group C at every time point examined. There were more brown granules in the intestinal crypts and epithelial cells compared to the other groups. The brown staining became faint and sparse in group E. The brown staining became dark and dense in group T. There was no significant difference in staining at different time points ($F = 1.269$, $P > 0.05$), although there were significant differences among groups ($F = 32.463$, $P < 0.05$). Further analysis showed significant differences between groups C and E and groups E and T ($P < 0.05$) (Figures 3-7).

IGF-1 mRNA expression in ileal tissue

IGF-1 mRNA was highly expressed in group C. There was no significant difference in expression at different times ($F = 0.086$, $P > 0.05$), although there were significant differences between groups ($F = 46.670$, $P < 0.05$). Further analysis revealed that there was a significant difference between groups C and E, groups C and T, and groups E and T ($P < 0.05$) (Figure 8).

DISCUSSION

Gastrointestinal dysfunction plays an important role in the development of multiple organ dysfunction syndrome (MODS). The intestine is one of the target organs injured in MODS, and it plays an important role in initiating MODS, in which intestinal mucosal barrier damage is a key factor. Bacterial or endotoxin translocation is closely related to excessive growth of opportunistic pathogens in the intestine, weakened intestinal immunity, and intestinal mucosal damage[3]. The intestinal flora is a key component of the mechanical, immune and biological barriers of the intestinal mucosa, as it plays a key role in preventing bacterial or endotoxin translocation.

Bifidobacterium is one of the main components of the intestinal mucosal barrier and is one of the main probiotics in the human body. It is involved in digestion, absorption, nutrition, metabolism, anti-infective immunity, and especially in maintaining the integrity of the intestinal mucosal barrier. The digestive tract is the largest bacterial habitat in the body. The micro-ecological stability of the intestine can be destroyed by fasting, antacids or antibiotics in critically ill patients, which can

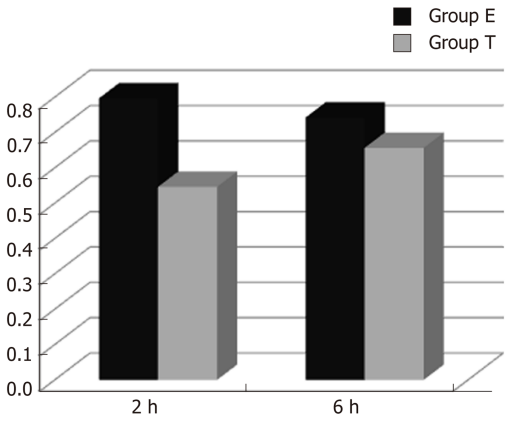


Figure 1 Optical density ratio of cytokine-induced neutrophil chemoattractant mRNA/β-actin mRNA in ileal tissue (mean ± SD).

disrupt the stability of the intestinal micro-ecology. Consequently, intestinal flora imbalance is the primary cause of bacterial translocation and intestinal infection. It has been reported that *Bifidobacterium* has an effect on rotavirus enteritis[4] and can prevent necrotizing enterocolitis in premature rats[5,6], where it has been shown to have a therapeutic effect on necrotizing enterocolitis[7,8]. *Bifidobacterium* also improves immunity and the inflammatory response in weaning rats with colitis[9], and there has been an increased research focus on the effects of *Bifidobacterium* on metabolic syndrome[10-15]. In recent years, *Bifidobacterium* has been used to prevent cardiac damage[16], and its effect on the immune state in early life has been studied[17]. However, the intestinal protective mechanism of *Bifidobacterium* has not been entirely elucidated.

In inflammatory reactions, neutrophils in the blood must be activated after entering tissue spaces through capillary walls, where they then exert their biological effects. Neutrophil chemotaxis is mediated by CINC, a murine chemotactic cytokine similar to human interleukin-8. CINC-1 expression is increased in rat models of ulcerative colitis[18], intestinal and lung ischemia/reperfusion models and endotoxin injury models[19,20]. CINC-1 expression in the lung tissue of rats is increased by endotoxin[20,21] and chronic intermittent hypoxia[22].

In the present study, there was no significant CINC mRNA expression in the ileum of group C rats at any time point examined. CINC mRNA expression was high at 2 and 6 h in group E, which meant that the model of intestinal injury was successful. CINC mRNA expression in the ileum of rats in group T was significantly decreased compared to group E ($P < 0.05$), which indicated that *Bifidobacterium* relieved ileal inflammation and protected the rat intestine.

IGF-1 is a multifunctional cellular regulatory factor that is mainly secreted by the liver. Thus, the liver is the main source of circulating IGF-1, but IGF-1 is also expressed in other tissues including the intestine, condylar cartilage cells in rats[23], T lymphocytes in mice dendritic epithelia[24], brain tissue of rats[25,26], placenta of pregnant mice[27-29], and colonic smooth muscle cells in diabetic rats[27]. IGF-1 plays a role in cell growth[30-32], differentiation[33,34] and metabolism[35]. One recent study focused on continuous IGF-1 expression in the intestine in a rat model of short bowel syndrome[36]. Other studies have shown that exogenous IGF-1 improved intestinal barrier function in rats with cirrhosis[37] or acute necrotizing pancreatitis[38], where survival rates have been significantly improved by minimal invasive interventions[39-43]. How IGF-1 is expressed during intestinal infection and how *Bifidobacterium* affects that expression have not been reported.

In this study, IGF-1 protein and mRNA expression in the ileum decreased after intraperitoneal injection of endotoxin, as reported previously[44]. Intragastric administration of *Bifidobacterium* increased IGF-1 protein and mRNA expression, indicating that IGF-1 plays an important role in the recovery of ileal mucosal damage. *Bifidobacterium* may enhance the immunological barrier function of the intestine by inhibiting local inflammatory responses and increasing IGF-1 expression.

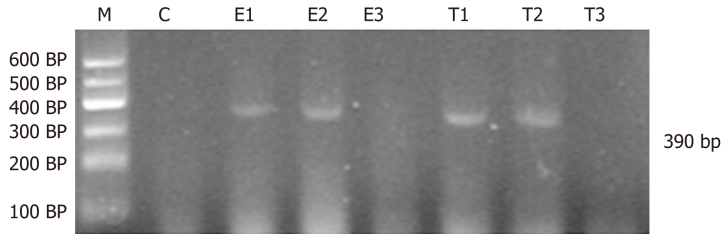


Figure 2 Cytokine-induced neutrophil chemoattractant mRNA expression in ileal tissue. M: DNA marker; C: Group C; E1–3: Group E 2 h, 6 h, and 12 h, respectively; T1–3: Group T 2 h, 6 h, and 12 h, respectively; 390 bp: Expected size of cytokine-induced neutrophil chemoattractant RT-PCR product.

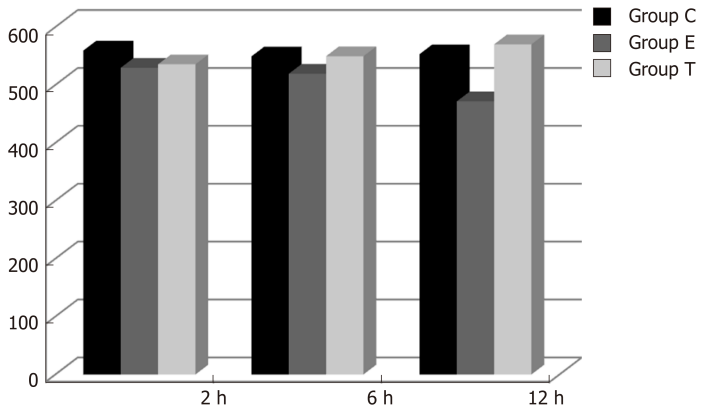


Figure 3 Immunohistochemical density of insulin-like growth factor 1 in the ileum (mean ± SD).

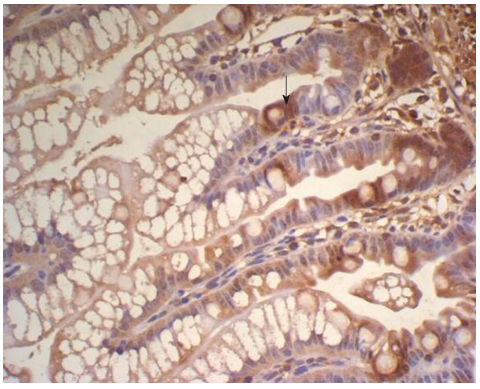


Figure 4 Insulin-like growth factor 1 expression in the ileum of group C, showing a large number of brown granules in the intestinal crypt and epithelial cells (× 400).

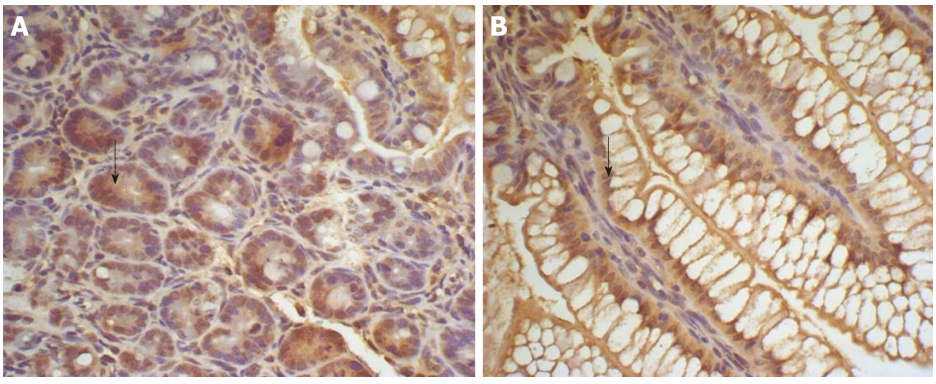


Figure 5 Expression of insulin-like growth factor 1 in the ileum of Group E and T at 2 h. A: Expression of insulin-like growth factor 1 in the ileum of group E at 2 h, showing many brown-staining granules in the intestinal epithelial cells (× 400); B: Expression of insulin-like growth factor 1 in the ileum of group T at 2 h, showing

many brown-staining granules in intestinal crypt and epithelial cells (× 400).

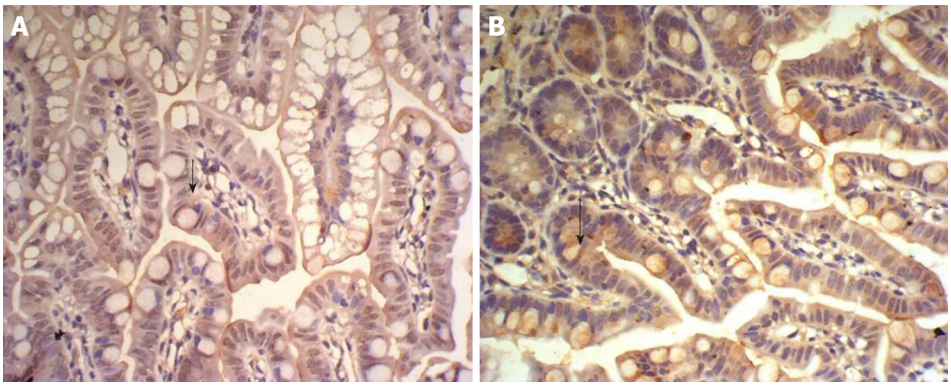


Figure 6 Expression of insulin-like growth factor 1 in the ileum of group E and T at 6 h. A: Expression of insulin-like growth factor 1 in the ileum of group E at 6 h, showing faint brown-staining granules in the intestinal epithelial cells (× 400); B: Expression of insulin-like growth factor 1 in the ileum of group T at 6 h, showing many densely distributed brown-staining granules in the intestinal crypt and epithelial cells (× 400).

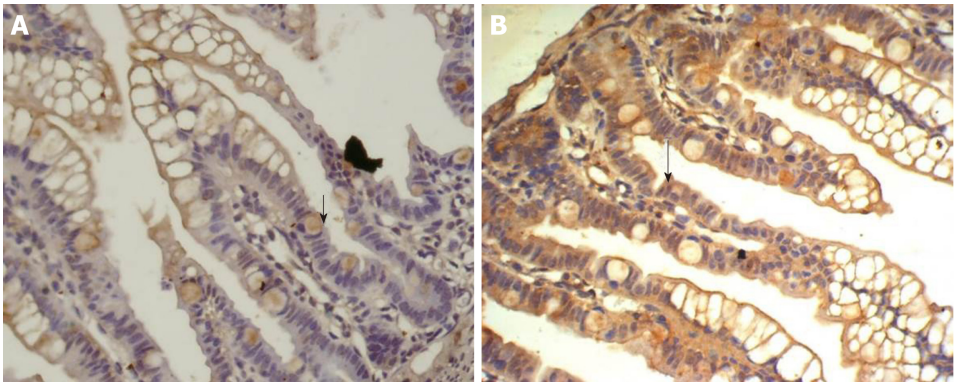


Figure 7 Expression of insulin-like growth factor 1 in the ileum of group E and T at 12 h. A: Expression of insulin-like growth factor 1 in the ileum of the group E at 12 h, showing faint and sparse brown-staining granules in the intestinal epithelial cells (× 400); B: Expression of insulin-like growth factor 1 in the ileum of group T at 12 h, showing diffuse and dense distribution of brown-staining granules in intestinal crypt and epithelial cells (× 400).

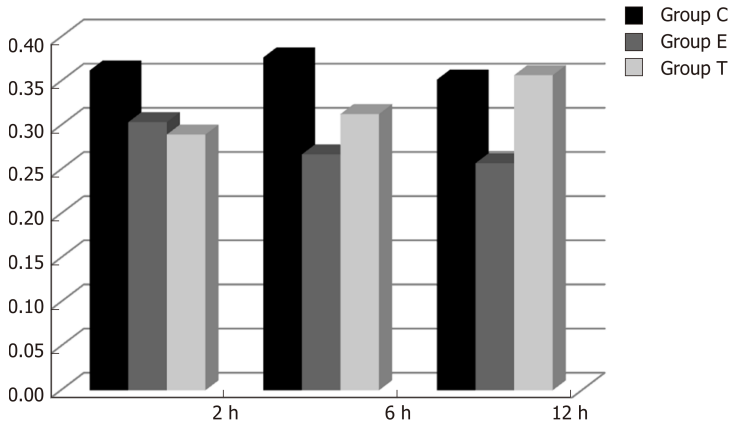


Figure 8 Optical density ratio of insulin-like growth factor 1 mRNA/β-actin mRNA in the ileum (mean ± SD).

ARTICLE HIGHLIGHTS

Research background

Severe infection is one of the most common causes of gastrointestinal dysfunction, and its

pathogenesis is closely related to endotoxemia and intestinal barrier injury.

Research motivation
Bifidobacterium plays an important role in maintaining the integrity of the intestinal mucosal barrier.

Research objectives
This study investigated the protective mechanism of *Bifidobacterium* during ileal injury in rats.

Research methods
Using endotoxin injured rat models, ileal cytokine-induced neutrophil chemoattractant (CINC) mRNA expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR), and expression of ileal insulin-like growth factor 1 (IGF-1) protein and mRNA was detected by immunohistochemistry and RT-PCR, respectively.

Research results
There was a significant difference in CINC mRNA expression between the different groups ($P < 0.05$). There was a significant difference in IGF-1 brown granule expression among the different groups ($P < 0.05$), and expression of IGF-1 mRNA significantly differed among the three groups ($P < 0.05$)

Research conclusions
Bifidobacterium may increase IGF-1 expression and enhance intestinal immune barrier function in rats with endotoxin injury.

Research perspectives
This study can provide a new therapeutic tool and theoretical support for gastrointestinal dysfunction.

REFERENCES

1 **Furusawa Y**, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 2013; **504**: 446-450 [PMID: 24226770 DOI: 10.1038/nature12721]

2 **Zhang R**, Shi Y, Guan X. Regulatory role of the GHSOCS2-IGF-1 axis in the pathogenesis of intestinal mucosal barrier dysfunction in ulcerative colitis. *Shijie Huaren Xiaohua Zazhi* 2010; **18**: 2442-2447 [DOI: 10.11569/wcjd.v18.i23.2442]

3 **Zhu R**, Ma XC. Clinical Value of Ultrasonography in Diagnosis of Pulmonary Embolism in Critically Ill Patients. *J Transl Int Med* 2017; **5**: 200-204 [PMID: 29340276 DOI: 10.1515/jtim-2017-0034]

4 **Rigo-Adrover M**, Saldaña-Ruíz S, van Limpt K, Knipping K, Garssen J, Knol J, Franch A, Castell M, Pérez-Cano FJ. A combination of scGOS/lcFOS with Bifidobacterium breve M-16V protects suckling rats from rotavirus gastroenteritis. *Eur J Nutr* 2017; **56**: 1657-1670 [PMID: 27112962 DOI: 10.1007/s00394-016-1213-1]

5 **Satoh T**, Izumi H, Iwabuchi N, Odamaki T, Namba K, Abe F, Xiao JZ. Bifidobacterium breve prevents necrotising enterocolitis by suppressing inflammatory responses in a preterm rat model. *Benef Microbes* 2016; **7**: 75-82 [PMID: 26420070 DOI: 10.3920/BM2015.0035]

6 **Wu SF**, Chiu HY, Chen AC, Lin HY, Lin HC, Caplan M. Efficacy of different probiotic combinations on death and necrotizing enterocolitis in a premature rat model. *J Pediatr Gastroenterol Nutr* 2013; **57**: 23-28 [PMID: 23535766 DOI: 10.1097/MPG.0b013e3182929210]

7 **Tang J**, Zou W, Li MX, Lv H, Huang LG, Yuan WM. Regulation of bifidobacterium on Wnt/β-Catenin signal pathway of intestinal tissue in newborn rats with necrotizing enterocolitis. *Zhonghua Shiyong Linchuang Erke Zazhi* 2016; **31**: 302-305 [DOI: 10.3760/cma.j.issn.2095-428X.2016.04.018]

8 **Su H**, Lv H, Zou W, Li MX, Huang LG, Li J, Yuan WM. Protective Effects of Biifdobacterium on intestinal tissue of newborn rats with necrotizing enterocolitis and its regulation. *Zhonghua Weichankexue Zazhi* 2015; **18**: 290-295 [DOI: 10.3760/cma.j.issn.1007-9408.2015.04.009]

9 **Izumi H**, Minegishi M, Sato Y, Shimizu T, Sekine K, Takase M. Bifidobacterium breve alters immune function and ameliorates DSS-induced inflammation in weanling rats. *Pediatr Res* 2015; **78**: 407-416 [PMID: 26083761 DOI: 10.1038/pr.2015.115]

10 **Zhu G**, Ma F, Wang G, Wang Y, Zhao J, Zhang H, Chen W. Bifidobacteria attenuate the development of metabolic disorders, with inter- and intra-species differences. *Food Funct* 2018; **9**: 3509-3522 [PMID: 29892745 DOI: 10.1039/c8fo00100f]

11 **Plaza-Díaz J**, Plaza-Díaz J, Robles-Sánchez C, Abadía-Molina F, Sáez-Lara MJ, Vilchez-Padial LM, Gil Á, Gómez-Llorente C, Fontana L. Gene expression profiling in the intestinal mucosa of obese rats administered probiotic bacteria. *Sci Data* 2017; **4**: 170186 [PMID: 29231922 DOI: 10.1038/sdata.2017.186]

12 **Plaza-Díaz J**, Robles-Sánchez C, Abadía-Molina F, Morón-Calvente V, Sáez-Lara MJ, Ruiz-Bravo A, Jiménez-Valera M, Gil Á, Gómez-Llorente C, Fontana L. Adamdec1, Ednrb and Ptgs1/Cox1, inflammation genes upregulated in the intestinal mucosa of obese rats, are downregulated by three probiotic strains. *Sci Rep* 2017; **7**: 1939 [PMID: 28512356 DOI: 10.1038/s41598-017-02203-3]

13 **Kim SJ**, Park SH, Sin HS, Jang SH, Lee SW, Kim SY, Kwon B, Yu KY, Kim SY, Yang DK. Hypocholesterolemic Effects of Probiotic Mixture on Diet-Induced Hypercholesterolemic Rats. *Nutrients* 2017; **9**: pii: E293 [PMID: 28300786 DOI: 10.3390/nu9030293]

14 **Plaza-Diaz J**, Gomez-Llorente C, Abadia-Molina F, Saez-Lara MJ, Campaña-Martin L, Muñoz-Quezada S, Romero F, Gil A, Fontana L. Effects of Lactobacillus paracasei CNCM I-4034, Bifidobacterium breve

CNCM I-4035 and Lactobacillus rhamnosus CNCM I-4036 on hepatic steatosis in Zucker rats. *PLoS One* 2014; **9**: e98401 [PMID: 24852284 DOI: 10.1371/journal.pone.0098401]
15 **Bordoni A**, Amaretti A, Leonardi A, Boschetti E, Danesi F, Matteuzzi D, Roncaglia L, Raimondi S, Rossi M. Cholesterol-lowering probiotics: *In vitro* selection and in vivo testing of bifidobacteria. *Appl Microbiol Biotechnol* 2013; **97**: 8273-8281 [PMID: 23872958 DOI: 10.1007/s00253-013-5088-2]
16 **Sadeghzadeh J**, Vakili A, Sameni HR, Shadnoush M, Bandegi AR, Zahedi Khorasani M. The Effect of Oral Consumption of Probiotics in Prevention of Heart Injury in a Rat Myocardial Infarction Model: A Histopathological, Hemodynamic and Biochemical Evaluation. *Iran Biomed J* 2017; **21**: 174-181 [PMID: 27874107 DOI: 10.18869/acadpub.ibj.21.3.174]
17 **Rigo-Adrover MD**, Franch À, Castell M, Pérez-Cano FJ. Preclinical Immunomodulation by the Probiotic Bifidobacterium breve M-16V in Early Life. *PLoS One* 2016; **11**: e0166082 [PMID: 27820846 DOI: 10.1371/journal.pone.0166082]
18 **Algieri F**, Zorrilla P, Rodriguez-Nogales A, Garrido-Mesa N, Bañuelos O, González-Tejero MR, Casares-Porcel M, Molero-Mesa J, Zarzuelo A, Utrilla MP, Rodriguez-Cabezas ME, Galvez J. Intestinal anti-inflammatory activity of hydroalcoholic extracts of Phlomis purpurea L. and Phlomis lychnitis L. in the trinitrobenzenesulphonic acid model of rat colitis. *J Ethnopharmacol* 2013; **146**: 750-759 [PMID: 23395625 DOI: 10.1016/j.jep.2013.01.041]
19 **Chen CB**, Tu WF, Xi WB. Effects of sufentanil pretreatment on long injury induced by ischemia-reperfusion hind limbs in Rats. *Linchuang Mazuixue Zazhi* 2015; **31**: 63-66
20 **Dong S**, Zhong Y, Yang K, Xiong X, Mao B. Intervention effect and dose-dependent response of tanreqing injection on airway inflammation in lipopolysaccharide-induced rats. *J Tradit Chin Med* 2013; **33**: 505-512 [PMID: 24187873 DOI: 10.1016/S0254-6272(13)60156-9]
21 **Sun YH**, Cui Y, Sun XJ, Li XQ. Effects of sevoflurane on neutrophil chemokine-1 and intercellular adhesion molecule-1 in endotoxin-induced acute lung injury in rats. *Guangdong Yixue* 2014; 35 [DOI: 10.13820/j.cnki.gdyx.2014.23.010]
22 **Han Q**, Li G, Mak JC, Zhang Y, Ip MS, Zhang N. [The role of heme oxygenase-1 in the protection of chronic intermittent hypoxia-induced lung injury *in vivo*]. *Zhonghua Jie He He Hu Xi Za Zhi* 2015; **38**: 516-519 [PMID: 26703018]
23 **Jiang LT**, Wei L, Xie YY, Zhou Q. Proliferation and Differentiation of Mandibular Condylar Chondrocytes Require IGF-1 Signal Pathway Related-proteins. *Kouqiang Hemian Waike Zazhi* 2013; **23**: 171-177
24 **Deng X**, Chen F, Liu J, Zhou Z, Jia C. [Expression of coxsackie-adenovirus receptor in keratinocytes of mouse skin after heat stimulation and the effect of coxsackie-adenovirus receptor on dendritic epidermal T lymphocytes]. *Zhonghua Shao Shang Za Zhi* 2014; **30**: 40-45 [PMID: 24684988]
25 **Zhang Y**, Shi B, Huang KX. Study on the protective effect of Mongolian treasure pill on cerebral tissue of neonatal rats with hypoxic brain injury. *Zhongguo Fuyou Baojian* 2014; **29**: 2596-2598
26 **Yan QF**, Chen YC, Huang SP, Heng XP, Chen Y, Li L, Wang SM, Kang WQ, Chen CY, Ye MJ, Zheng HL. Dangua recipe on intervening IGF-1 in encephalon of mice with Apo E-/-Diabetes Mellitus. *Jilin Zhongyiyao Zazhi* 2015; **35**: 404-406
27 **Zhang YD**, Liang JW, Wang J, Liang B. Pregnant rats intake different levels of glucose to influence the placental IGFs expression. *Zhonghua Linchuang Yishi Zazhi* 2015; **9**: 470-474
28 **Wu MM**, Yang F, Qu Y, Mu DZ. [Effects of maternal folate deficiency on the methylation of insulin-like growth factor system in the offspring rats]. *Zhongguo Dang Dai Er Ke Za Zhi* 2017; **19**: 470-474 [PMID: 28407838]
29 **Wang Y**, Xu XY, Tang YR, Yang WW, Yuan YF, Ning YJ, Yu YJ, Lin L. Effect of endogenous insulin-like growth factor and stem cell factor on diabetic colonic dysmotility. *World J Gastroenterol* 2013; **19**: 3324-3331 [PMID: 23745035 DOI: 10.3748/wjg.v19.i21.3324]
30 **Wang DH**, Qi HX, Luo KJ, Chen PY. Effects of puerarin subcutaneous injection on body and bone metabolism in pregnant rats on low-protein diet during pregnancy. *Shangdong Yiyao* 2017; **57**: 46-48
31 **Wu WY**, Wang JY, Xu XJ. Body mass catch-up growth in premature infants and infants less than gestational age and its correlation with igf-1. *Shangdong Yiyao* 2015; **1**: 69-71
32 **Han H**, Li Y. Effect of rat nerve growth factor on nerve conduction velocity and serum IGF-1in patients with diabetic peripheral neuropathy. *Zhongguo Shiyong Yikan* 2014; **41**: 85-87
33 **Jiang ZM**, Lu DX, Li HX. Therapeutic effect of mouse nerve growth factor on acute cerebral infarction. *Shandong Yiyao* 2015; **34**: 106-107
34 **Yan QY**, Li Z, Hu WT, Fang Y, Wang SN, Qiu ZD, Zhang SM. Roles of Insulin-like Growth Factor-1 in the Induction of Murine Induced Pluripotent Stem Cells. *Shengjing Sunshang Yu Gongneng Chongjian* 2013; **8**: 157-159
35 **Dong X**, Chang G, Ji XF, Tao DB, Wang YX. The relationship between serum insulin-like growth factor I levels and ischemic stroke risk. *PLoS One* 2014; **9**: e94845 [PMID: 24728374 DOI: 10.1371/journal.pone.0094845]
36 **Sangild PT**, Ney DM, Sigalet DL, Vegge A, Burrin D. Animal models of gastrointestinal and liver diseases. Animal models of infant short bowel syndrome: Translational relevance and challenges. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G1147-G1168 [PMID: 25342047 DOI: 10.1152/ajpgi.00088.2014]
37 **Zhao TY**, Su LP, Ma CY, Zhai XH, Duan ZJ, Zhu Y, Zhao G, Li CY, Wang LX, Yang D. IGF-1 decreases portal vein endotoxin via regulating intestinal tight junctions and plays a role in attenuating portal hypertension of cirrhotic rats. *BMC Gastroenterol* 2015; **15**: 77 [PMID: 26152281 DOI: 10.1186/s12876-015-0311-5]
38 **Cui PL**, Lv D, Wang YB, Yang ZX, Xu YQ. Effects of glutamine on expression of IGF-1 and intestinal mucosal barrier in rats with acute necrotic pancreatitis. *Shandong Yiyao* 2014; **54**: 14-17
39 **Adler DG**. Single-operator experience with a 20-mm diameter lumen apposing metal stent to treat patients with large pancreatic fluid collections from pancreatic necrosis. *Endosc Ultrasound* 2018; **7**: 422-423 [PMID: 30531025 DOI: 10.4103/eus.eus_39_18]
40 **Poincloux L**, Chabrot P, Mulliez A, Genes J, Boyer L, Abergel A. Interventional endoscopic ultrasound: A new promising way for intrahepatic portosystemic shunt with portal pressure gradient. *Endosc Ultrasound* 2017; **6**: 394-401 [PMID: 29251274 DOI: 10.4103/eus.eus_42_17]
41 **Adler DG**, Shah J, Nieto J, Binmoeller K, Bhat Y, Taylor LJ, Siddiqui AA. Placement of lumen-apposing metal stents to drain pseudocysts and walled-off pancreatic necrosis can be safely performed on an outpatient basis: A multicenter study. *Endosc Ultrasound* 2019; **8**: 36-42 [PMID: 29770780 DOI: 10.4103/eus.eus_30_17]

42 **Huang JY**. Role of EUS-guided liver biopsy in benign parenchymal disease (with video). *Endosc Ultrasound* 2018; **7**: 236-239 [PMID: 30117485 DOI: 10.4103/eus.eus_33_18]
43 **Rana SS**, Gupta R, Kang M, Sharma V, Sharma R, Gorsi U, Bhasin DK. Percutaneous catheter drainage followed by endoscopic transluminal drainage/necrosectomy for treatment of infected pancreatic necrosis in early phase of illness. *Endosc Ultrasound* 2018; **7**: 41-47 [PMID: 29451168 DOI: 10.4103/eus.eus_94_17]
44 **He Y**, Yuan X, Zhou G, Feng A. Activation of IGF-1/IGFBP-3 signaling by berberine improves intestinal mucosal barrier of rats with acute endotoxemia. *Fitoterapia* 2018; **124**: 200-205 [PMID: 29154866 DOI: 10.1016/j.fitote.2017.11.012]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2935-2946

DOI: 10.3748/wjg.v25.i23.2935

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Case Control Study

Plasma Nogo-A and placental growth factor levels are associated with portal hypertension in patients with liver cirrhosis

Sigita Gelman, Violeta Salteniene, Andrius Pranculis, Jurgita Skieceviciene, Romanas Zykus, Dalius Petrauskas, Limas Kupcinskas, Ali Canbay, Alexander Link, Juozas Kupcinskas

ORCID number: Sigita Gelman (0000-0002-7324-1425); Violeta Salteniene (0000-0002-7881-945X); Andrius Pranculis (0000-0002-3772-0867); Jurgita Skieceviciene (0000-0002-4893-6612); Romanas Zykus (0000-0001-7204-0106); Dalius Petrauskas (0000-0003-3756-6238); Limas Kupcinskas (0000-0002-8689-9023); Ali Canbay (0000-0001-6069-7899); Alexander Link (0000-0002-9514-4562); Juozas Kupcinskas (0000-0002-8760-7416).

Author contributions: Kupcinskas L and Kupcinskas J designed the research; Gelman S, Zykus R and Petrauskas D treated patients, collected material and clinical data from the patients; Salteniene V and Skieceviciene J performed the assays, Pranculis A performed hepatic vein catheterization and HVPG measurement, Gelman S analyzed the data and drafted the manuscript, Canbay A, Link A and Kupcinskas J approved the final version of the manuscript .

Supported by the Research Fund of Lithuanian University of Health Sciences (SV5-074/BN17-99), No. LSMU-21.

Institutional review board statement: This study was approved by the institutional review board of Lithuanian University of Health Sciences.

Informed consent statement: Informed consent was obtained from each patient.

Conflict-of-interest statement: The

Sigita Gelman, Romanas Zykus, Dalius Petrauskas, Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Violeta Salteniene, Jurgita Skieceviciene, Institute for Digestive Research, Medical Academy, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Andrius Pranculis, Department of Radiology, Medical Academy, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Limas Kupcinskas, Juozas Kupcinskas, Institute for Digestive Research and Department of Gastroenterology, Medical Academy, Lithuanian University of Health Sciences, Kaunas 44307, Lithuania

Ali Canbay, Alexander Link, Department of Gastroenterology, Hepatology and Infectious Diseases Otto-von-Guericke University, Magdeburg 39106, Germany

Corresponding author: Juozas Kupcinskas, MD, PhD, Doctor, Professor, Research Fellow, Senior Researcher, Senior Scientist, Institute for Digestive Research, Lithuanian University of Health Sciences, A. Mickeviciaus Str. 9, Kaunas 44307, Lithuania.
juozas.kupcinskas@lsmuni.lt
Telephone: +370-37-326898

Abstract

BACKGROUND

Clinically significant portal hypertension (CSPH) and severe portal hypertension (SPH) increase the risk for decompensation and life-threatening complications in liver cirrhosis. Pathologic angiogenesis might contribute to the formation of these conditions. Placental growth factor (PlGF) and Nogo-A protein are biomarkers of pathological angiogenesis, but data on their role in liver cirrhosis and portal hypertension is scarce.

AIM

To determine plasma levels of PlGF and Nogo-A in patients with liver cirrhosis, CSPH, SPH and potential to predict portal hypertension.

METHODS

A cohort of 122 patients with hepatitis C virus and/or alcohol-induced liver cirrhosis with characterized hepatic venous pressure gradient (HVPG) were included in the study. Demographic data, medical history, Child-Turcotte-Pugh and Model of End Stage liver disease score, clinical chemistry, liver stiffness

authors have declared no conflicts of interest.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Manuscript source: Unsolicited manuscript

Received: March 23, 2019
Peer-review started: March 25, 2019
First decision: April 11, 2019
Revised: April 18, 2019
Accepted: April 29, 2019
Article in press: April 29, 2019
Published online: June 21, 2019

P-Reviewer: Garbuzenko DV, Gencdal G, Swierczynski JT, Yoshioka K
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

values were recorded on the day of the procedure prior HVPG measurement. The degree of portal hypertension was determined by the invasive HVPG measurement. Nogo-A and PlGF plasma levels were evaluated using enzyme linked immunosorbent assay. The control group consisted of 30 healthy age- and sex- matched individuals.

RESULTS

Peripheral PlGF levels were higher and Nogo-A levels were lower in patients with liver cirrhosis (23.20 *vs* 9.85; $P < 0.0001$ and 2.19 *vs* 3.12; $P = 0.004$ respectively). There was a positive linear correlation between peripheral levels of PlGF and HVPG ($r = 0.338$, $P = 0.001$) and negative linear correlation between the peripheral Nogo-A levels and HVPG ($r = -0.267$, $P = 0.007$). PlGF levels were higher in CSPH and SPH ($P = 0.006$; $P < 0.0001$) whereas Nogo-A levels were lower ($P = 0.01$; $P < 0.033$). Area under the curve for the diagnosis of CSPH for PlGF was 0.68 ($P = 0.003$) and for Nogo-A - 0.67 ($P = 0.01$); for SPH 0.714 ($P < 0.0001$) and 0.65 ($P = 0.014$) respectively. PlGF levels were higher and Nogo-A levels were lower in patients with esophageal varices ($P < 0.05$). PlGF cut-off value of 25 pg/mL distinguished patients with CSPH at 55.7% sensitivity and 76.7% specificity; whereas Nogo-A cut-off value of 1.12 ng/mL was highly specific (93.1%) for the diagnosis of CSPH.

CONCLUSION

Plasma PlGF levels were higher while Nogo-A levels were lower in patients with liver cirrhosis and portal hypertension. Biomarkers showed moderate predictive value in determining CSPH and SPH.

Key words: Liver cirrhosis; Portal hypertension; Angiogenesis; Placental growth factor; Nogo-A; Hepatic venous pressure gradient

Core tip: In this study, we aimed to evaluate plasma levels of angiogenesis mediators placental growth factor and Nogo-A protein in patients with liver cirrhosis, clinically significant portal hypertension, severe portal hypertension as well as biomarker potential to predict clinically significant and severe portal hypertension. Higher levels of placental growth factor have previously been associated with portal hypertension in animal models; however, data in patients with liver cirrhosis are scarce. To date this is the first study to evaluate Nogo-A protein levels in patients with liver cirrhosis and portal hypertension. Furthermore, to our best knowledge this is the first study to evaluate prognostic potential of these biomarkers to detect clinically significant and severe portal hypertension. We believe that this study adds additional knowledge on the complex pathogenesis of portal hypertension and might provide new insights for future research of new diagnostic approaches and treatment targets in the field.

Citation: Gelman S, Salteniene V, Pranculis A, Skieceviciene J, Zykus R, Petrauskas D, Kupcinskas L, Canbay A, Link A, Kupcinskas J. Plasma Nogo-A and placental growth factor levels are associated with portal hypertension in patients with liver cirrhosis. *World J Gastroenterol* 2019; 25(23): 2935-2946
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2935.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2935

INTRODUCTION

Portal hypertension (PH) is a consequence of liver cirrhosis and can cause serious life-threatening complications. The degree of portal hypertension is one of the most important prognostic factors for complications and decompensation of liver cirrhosis and is defined by the hepatic venous pressure gradient (HVPG)[1]. The presence of clinically significant portal hypertension (CSPH; HVPG ≥ 10 mmHg) increases the risk for the formation of varicose veins and decompensation whereas severe portal hypertension (SPH; HVPG ≥ 12 mmHg) increases the risk for variceal bleeding and

death[1-3]. In order to optimize the care of patients with liver cirrhosis, it is essential to detect early PH and prevent the development of CSPH or treat already present CSPH to avoid decompensation[4]. As current options for the prevention and treatment of CSPH are limited to attenuating splanchnic vasodilatation, new insights and deeper knowledge about pathogenetic me-chanisms of PH and molecules involved, would aid in search for newer and more effective treatment strategies[5].

The main pathophysiological mechanism of PH in cirrhosis is an increase in intrahepatic vascular resistance (IHVR), mostly caused by structural changes in liver tissue, but functional disorders of liver circulation are also important pathogenetic factors[1]. Recent studies suggest that pathologic angiogenesis could also contribute to an increase in IHVR, causing splanchnic hyperemia, portosystemic collateralization and pathologic angiogenesis inside the liver, worsening an already existing PH[5-7].

Pathologic angiogenesis has been reported to result from the upregulation of proangiogenic factors and simultaneous downregulation of angiogenesis inhibitors, thus both stimuli might be important and contribute to the pathophysiological mechanisms[8].

Vascular endothelial growth factor (VEGF) family has been recognized as one of the major proangiogenic mediators associated with PH[9] in number of animal studies[10-12] and only two small human studies[13,14] demonstrating beneficial effects on PH when blocking VEGF signaling. However this could cause deleterious side effects, as angiogenesis is essential for tissue healing and regeneration[5]. Placental growth factor (PlGF), a member of the VEGF family, on the other hand could be a more promising target as it has been reported only to enhance angiogenesis in pathological conditions, not affecting normal tissues[15]. As a porangiogenic factor PlGF escalates the proliferation, migration and survival of the endothelial cells, intensifies the proliferation of the mesenchymal cells and regulates the contraction of the mural cells[16]. The expression and function of PlGF has been addressed in several animal studies[17,18], however data on plasma levels of PlGF in patients with liver cirrhosis, portal hypertension and it's complications is scarce.

Angiogenesis is a two way process including activation and inhibition of angiogenesis stimuli and a number of endogenous angiogenesis inhibitors have been identified, some of them being associated with PH[19,20]. Nogo-A and Nogo-B proteins are novel recently discovered angiogenesis mediators that belong to the reticulon 4 protein family. Nogo-A protein, most widely examined as a potent neurite growth inhibitor, has never been studied in patients with liver cirrhosis or PH. Studies have examined the expression of Nogo-A in cardiomyocytes[21], enteric nervous system[22], in ocular diseases[23] and hepatocellular carcinoma[24]. Nogo-A has also been reported to be a negative regulator of retinal and CNS[25,26] angiogenesis, but the effects on angiogenesis in liver diseases remains unknown. As Nogo-B protein, a splice variant of Nogo-A, is associated with liver fibrogenesis and liver cirrhosis[27,28], angiogenesis[29,30] and endogenous tissue repair[31], we hypothesized that Nogo-A, similarly to Nogo-B, might be associated with liver cirrhosis and the pathogenesis of PH.

In this study we aimed to evaluate plasma levels of PlGF and Nogo-A in liver cirrhosis patients with normal portal pressure and portal hypertension as well as in controls. We also aimed to evaluate the potential of plasma PlGF and Nogo-A levels as biomarkers to predict CSPH and SPH as well as complications of portal hypertension.

MATERIALS AND METHODS

Patients

The study included patients with hepatitis C virus and/or alcohol induced liver cirrhosis who underwent a scheduled HVPG measurement or transjugular liver biopsy in the Department of Gastroenterology, Lithuanian University of Health Sciences from September 2015 to December 2017. A total of 290 patients were examined, out of which 122 were included in the study. The main criteria for the exclusion from the study were: Pre- or posthepatic causes of portal hypertension, cardiovascular, kidney disease, diabetes, neurodegenerative diseases, active infection, hepatocellular carcinoma or cancer of other location. We also excluded the patients with the history of current use of beta-blockers or other vasoactive drugs. Demographic data, medical history (presence of ascites, esophageal varices), Child-Turcotte-Pugh and Model of End Stage liver disease (MELD) score, clinical chemistry, liver stiffness (Fibroscan, Echosens, France), plasma levels of Nogo-A and PlGF, HVPG values were recorded on the day of the procedure prior HVPG measurement.

Liver cirrhosis was diagnosed according to clinical, laboratory and radiologic data and/or histology; the presence of portal hypertension was diagnosed by hepatic vein

catheterization.

Thirty healthy volunteers, matched for age and sex, with normal liver enzymes and normal liver stiffness measurements were included as controls for peripheral levels of Nogo-A and PlGF.

The study was approved by Kaunas Region Biomedical Research Ethics Committee (2015-08-24, No. BE-2-28, Kaunas, Lithuania). Every participant provided a written informed consent to participate in the study and study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Nogo-A and PlGF measurements

Peripheral blood samples were obtained on the day of the procedure. Blood samples from the hepatic vein were obtained during the HVPG measurement in EDTA tubes. Samples were centrifuged at room temperature in 3500 g for 10 min, plasma was separated and stored in -80 °C for further analysis. Sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to determine Nogo-A (Elabscience, United States) and PlGF (Abbexa LTD, United Kingdom) plasma levels, according to manufacturers' protocols using Sunrise™ (Tecan Trading AG, Switzerland) microplate reader with 450 nm wavelength filter and Magellan™ (Tecan Trading AG, Switzerland) software. In each ELISA kit both controls and patients were included.

HVPG measurement

The degree of portal hypertension was determined by the invasive HVPG measurement. The procedure was performed by the same experienced radiologist according to the standard as described by Groszmann *et al*[32]. At least three repeated measurements were performed to determine free and wedged hepatic vein pressure for calculation of HVPG. HVPG values of 1-5 mmHg were considered to represent normal portal pressure, whereas portal hypertension was diagnosed at a HVPG ≥ 6 mmHg. HVPG ≥ 10 mmHg was considered to be CSPH and ≥ 12 mmHg-SPH.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 software. Descriptive statistics are provided as mean and standard deviation (SD), or as median and range for non-parametric data. Differences between the groups were assessed with the Student's *t* test or Mann-Whitney's test as appropriate. Differences between three groups were assessed by one-way ANOVA test or the Wilcoxon test, when appropriate. Correlations were performed by means of Spearman's correlation for PlGF and Pearson's correlation for Nogo-A and expressed by Spearman's or Pearson's coefficient. Univariate regression analysis was performed to identify the relationship of PlGF and Nogo-A with PH and its complications. Receiver operating characteristic (ROC) curves were created to assess the predictive values of PlGF and Nogo-A for CSPH, SPH and complications with area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The value with the best sensitivity and specificity in AUC analysis (Youden's Index) was chosen for further analysis. Statistical significance was established at $P < 0.05$ and expressed as $^{a}P < 0.05$.

RESULTS

Patient characteristics

One hundred and twenty-two patients with liver cirrhosis and 30 controls were included in the study. Sixty-seven patients (54.9%) were male and mean age was 50.45 (± 11.13). Sixty-one (50%) patient had alcohol induced liver cirrhosis and sixty-one (50%)-hepatitis C virus cirrhosis. Seventy-five (61.5%) patients were classified as having Child-Pugh A, 30 (24.6%) as Child-Pugh B and 17 (13.9%) as Child-Pugh C cirrhosis. Fourteen patients (11.5%) had normal HVPG, eighty-six (70.5 %) had HVPG ≥ 10 mmHg, of which 70 (57.4%) had HVPG ≥ 12 mmHg. Demographic, clinical and endoscopic characteristics are displayed in Table 1. Peripheral Nogo-A and PlGF levels were examined in 100 patients each and hepatic Nogo-A and PlGF levels were examined in 30 patients each.

PlGF and Nogo-A plasma levels in liver cirrhosis

Median peripheral PlGF levels were higher in patients with liver cirrhosis when compared to controls (23.20; range 14.08-37.70 pg/mL *vs* 9.85; range 4.88-22.72 pg/mL; $P < 0.0001$; Figure 1). Peripheral PlGF levels were higher in patients with alcohol-induced cirrhosis when compared to patients with hepatitis C virus cirrhosis (29.10; range 16.52-41.71 pg/mL *vs* 20.92; range 13.72-34.42 pg/mL; $P = 0.049$) and both were significantly higher than in controls (9.85; range 4.88-22.72 pg/mL). Levels

Table 1 Demographic, clinical and endoscopic characteristics of the patients and controls

Variable	Patients (*n* = 122)	Controls (*n* = 30)
Sex (male/female; %)	55/45	50/50
Age (yr; SD)	51.14 (± 9.73)	48.92 (± 15.46)
BMI (kg/m^2; SD)	26.04 (± 4.63)	25.30 (± 3.51)
Etiology (N (%) of patients)		
Alcohol induced cirrhosis	61 (50)	
HCV cirrhosis	61 (50)	
Child-Pugh class (A/B/C; %)	61.5/24.6/13.9	
MELD score (SD)	11.15 (± 4.32)	
TE (kPa; SD)	31.37 (± 19.79)	
Ascites (% of patients)	38.5	
Varices (% of patients)		
Absent	45.1	
F1	27	
F2	18.9	
F3	9	
Risk signs of bleeding (N of patients)	24	
HVPG (mmHg; SD)	13.70 (± 6.52)	
HVPG 1-5 mmHg (% of patients)	11.5	
HVPG 5-9 mmHg (% of patients)	19.7	
CSPH; HVPG ≥ 10 mmHg (% of patients)	70.5	
SPH; HVPG ≥ 12 mmHg (% of patients)	57.4	

SD: Standard deviation; BMI: Body mass index; HCV: Hepatitis C virus; MELD: Model of End Stage Liver Disease; HVPG: Hepatic venous pressure gradient; CSPH: Clinically significant portal hypertension; SPH: Severe portal hypertension; TE: Transient elastography.

of PlGF at the hepatic vein did not differ significantly from those at the peripheral vein (25.22 pg/mL, range 10.51-34.73 *vs* 21.22 pg/mL, range 9.48-44.87; $P = 0.289$). There was a positive linear correlation between the peripheral PlGF levels and Child-Pugh score ($r = 0.424$; $P < 0.0001$). Peripheral PlGF levels were increasing with the Child-Pugh stage: in patients with Child-Pugh class A PlGF was 19.64 pg/mL (IQR: 13.38-31.42 pg/mL), in Child-Pugh class B 29.65 pg/mL (IQR: 18.95-46.29 pg/mL) and in Child-Pugh class C 32.80 pg/mL (IQR: 28.00-49.93 pg/mL). PlGF levels were significantly higher in Child-Pugh class B ($P < 0.042$) and class C ($P < 0.002$) when compared to Child-Pugh class A. Positive linear correlation was also observed with MELD score ($r = 0.283$; $P < 0.006$). PlGF levels at the hepatic vein correlated with Child-Pugh score ($r = 0.384$; $P = 0.036$) and were significantly higher in Child-Pugh C stage ($P = 0.007$), when compared to Child-Pugh A and B stage.

Mean peripheral mean levels of Nogo-A protein were lower in patients with liver cirrhosis (2.19 ± 1.47 ng/mL) when compared to controls (3.12 ± 1.54 ng/mL; $P = 0.004$; Figure 2). Peripheral Nogo-A protein levels did not differ between the alcohol-induced (2.22 ± 1.59 ng/mL) and hepatitis C virus cirrhosis (2.02 ± 1.33 ng/mL; $P = 0.785$), however both were significantly lower than in controls (3.15 ± 1.48 ng/mL). Peripheral levels of Nogo-A were significantly lower when compared to the levels at the hepatic vein (1.70 ± 1.19 ng/mL *vs* 3.48 ± 2.27 ng/mL respect. $P < 0.0001$). No significant correlation was observed between peripheral and hepatic Nogo-A levels and Child-Pugh score as well as MELD score. Peripheral Nogo-A levels did not differ between the Child-Pugh stage groups (Child-Pugh A 2.19 ± 1.37 ng/mL; Child-Pugh B 1.9 ± 1.49 ng/mL and Child-Pugh C 2.17 ± 1.84 ng/mL).

To evaluate the relationship of plasma PlGF and Nogo-A levels with liver stiffness we calculated the correlation of biomarkers with values of transient elastography (TE; Fibroscan). Transient elastography measurements were available for 74 patients. There was a positive linear correlation between peripheral PlGF values and TE ($r = 0.364$; $P < 0.001$), but hepatic levels of PlGF did not correlate with liver stiffness. Peripheral Nogo-A levels did not correlate with liver stiffness.

PlGF and Nogo-A plasma levels in portal hypertension

There was a positive linear correlation between peripheral levels of PlGF and HVPG ($r = 0.338$, $P = 0.001$, Figure 3). Hepatic PlGF levels did not correlate with HVPG.

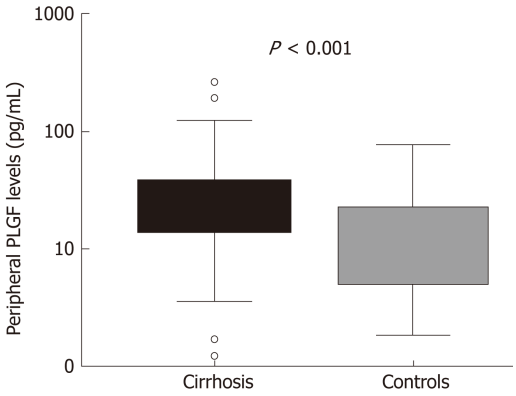


Figure 1 Peripheral placental growth factor levels in liver cirrhosis and controls. Box plot graph; boxes correspond to the median value and interquartile range. PlGF: Placental growth factor.

Higher peripheral PlGF values were significantly associated with ascites (OR = 5.59; *P* = 0.018). Linear regression revealed that an increase of PlGF by 10 points increased the HVPG by 0.5 mmHg (*P* = 0.003).

Peripheral levels of PlGF were significantly higher in patients with CSPH (28.20 pg/mL; range 17.09-41.17 pg/mL) than in patients without CSPH (17.20 pg/mL; range 10.12-26.00 pg/mL; *P* = 0.006), as well as in patients with SPH (29.27 pg/mL; range 19.83-42.68 pg/mL) when compared to patients without SPH (15.39 pg/mL; range 10.15-24.99 pg/mL; *P* < 0.0001). AUC for the diagnosis of CSPH was 0.68 (CI: 0.56-0.79; *P* = 0.003 Figure 4A) and for the diagnosis of SPH 0.714 (CI: 0.61-0.82; *P* < 0.0001 Figure 4B). A cut-off value of 25 pg/mL provided the most accurate sensitivity and specificity to discriminate between patients with and without CSPH, whereas a cut-off value of 26.8 pg/mL helped to differentiate between the patients with and without SPH (Table 2). When classifying patients according to the cut-off values, OR for the presence of CSPH was 4.13 (*P* = 0.004) and for the presence of SPH 5.58 (*P* < 0.0001).

There was a negative linear correlation between the peripheral Nogo-A levels and HVPG (*r* = -0.267, *P* = 0.007, Figure 5). Hepatic Nogo-A levels did not correlate with HVPG. Linear regression showed an increase of HVPG by 1.2 mmHg per decrease of Nogo-A levels by 1 point (*P* = 0.007).

Peripheral levels of Nogo-A were significantly lower in patients with CSPH (1.96 ± 1.39 ng/mL) than in patients without CSPH (2.77 ± 1.53 ng/mL; *P* = 0.011), as well as in patients with SPH (1.94 ± 1.46 ng/mL) when compared to patients without SPH (2.57 ± 1.42 ng/mL; *P* < 0.033). AUC for the diagnosis of CSPH was 0.67 (CI: 0.55-0.78; *P* = 0.01 Figure 6A) and for the diagnosis of SPH 0.65 (CI: 0.54-0.75; *P* = 0.014 Figure 6B). A cut-off value of 1.12 ng/mL yielded the most accurate sensitivity and specificity to differentiate between patients with and without CSPH whereas a cut-off value of 1 ng/mL helped to differentiate between the patients with and without SPH (Table 2). When classifying patients according to the cut-off values, OR for the presence of CSPH was 7.8 (*P* = 0.008) and for the presence of SPH 6.12 (*P* = 0.006).

PlGF and Nogo-A plasma levels and esophageal varices

Both peripheral PlGF and Nogo-A were significantly associated with esophageal varices. Peripheral PlGF values were higher and Nogo-A levels were lower in patients with esophageal varices when compared to patients without esophageal varices (PlGF 29.96 pg/mL; range 20.34-44.03 *vs* 17.73 pg/mL; range 11.76-27.97; *P* = 0.001; and Nogo-A 1.94 ± 1.48 ng/mL *vs* 2.52 ± 1.41 ng/mL; *P* < 0.05, respectively). The PlGF cut-off value of 19.83 pg/mL and Nogo-A cut-off value of 2.3 ng/mL were the most sensitive and specific in order to discriminate patients having esophageal varices. When categorizing patients according to peripheral PlGF levels above 19.83 pg/mL, the OR for the presence of esophageal varices was 5.25 (*P* = 0.0001). With peripheral Nogo-A levels below 2.3 ng/mL, the OR for the presence of esophageal varices was 3.2 (*P* = 0.007). However, the levels of biomarkers did not differ between different grades of esophageal varices (Figures 1-3).

To evaluate the esophageal varices with high risk of bleeding we selected a separate group of patients with large (Figure 3) esophageal varices and with varices containing red wale marks or cherry red spots on endoscopy (*n* = 21). Higher peripheral PlGF levels were associated with high risk esophageal varices (OR = 7.512; *P* = 0.01). AUC for the presence of high-risk esophageal varices was 0.62 (CI: 0.54-0.77) (Table 2).

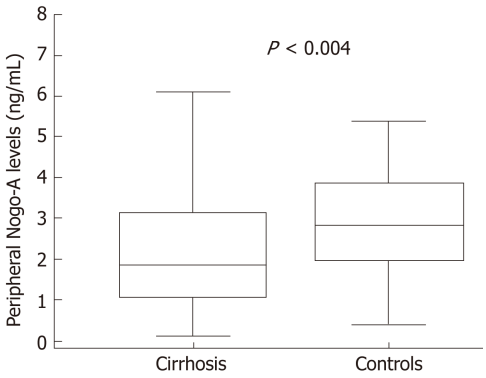


Figure 2 Peripheral Nogo-A levels in liver cirrhosis and controls. Box plot graph; boxes correspond to the mean value and standard deviation.

DISCUSSION

With growing burden on chronic liver diseases worldwide[33], the optimization of care provided to the patients with liver cirrhosis has become a priority. According to the Baveno VI consensus meeting the detection and treatment of early PH as well as the prevention and timely treatment of CSPH, in order to avoid complications and decompensation, are of utmost importance[4]. The consensus has encouraged the search for new treatment strategies to target different pathogenetic components of PH[4].

This prospective study was aimed to gain deeper knowledge on the molecules involved in the abnormal angiogenesis-one of the pathogenetic components of PH. We have demonstrated differences of proangiogenic mediator PlGF and angiogenesis inhibitor Nogo-A plasma levels in patients with liver cirrhosis, when compared to controls. We have also determined a clear correlation of these mediators to PH and complications of PH. Furthermore, we have for the first time demonstrated that plasma concentration of Nogo-A is lower in liver cirrhosis and PH, as well as the predictive values of plasma PlGF and Nogo-A levels in diagnosing CSPH, SPH and PH complications. This information provides further insights in the pathogenesis of PH in liver cirrhosis and might be useful in creating new noninvasive diagnostic models for CSPH, as it is one of the most important prognostic factors for patients with liver cirrhosis.

Data of recent research suggests that angiogenesis plays an important role in the development of PH by contributing to all pathogenetic processes involved in the formation of PH and its complications: An increased intra-hepatic resistance due to the establishment of abnormal liver angioarchitecture, portosystemic collateralization and possibly development and maintenance of splanchnic hyperemia as well as hyperdynamic circulation[7,15,20]. This evidence supporting the role of angiogenesis in the pathogenesis of PH arises from the animal and human studies involving a potent proangiogenic factor-VEGF. VEGF has been shown to be overexpressed in the splanchnic circulation in animal models of PH[11,12], an increased VEGF mediated angiogenesis was observed in animal models as well as cirrhotic patients with PH[34,35].

PlGF is a member of VEGF family, which is upregulated in pathological tissues and almost undetectable in healthy tissues[16], making it a perspective candidate for the therapeutic strategies. PlGF has been identified by multiple studies as a disease modifying agent in various organs and organ systems, including heart, muscles, skin, solid tumors, nervous system, colon, lungs[16]. In liver PlGF has been found to be associated with inflammation, fibrosis, angiogenesis[17,18] and portal hypertension[36-38]. The role of PlGF in portal hypertension is believed to be associated with pathological angiogenesis and vascular maturing through the action on VEGFR-1 receptor[5,20]. Thus PlGF antibodies or PlGF knockout decreased neo-vascularization, splanchnic blood flow and portal pressure in animal models[9,17,39]. To our knowledge only one previous study evaluated the association of PlGF to liver cirrhosis and HVPG in humans. Van Steenkiste *et al*[17] reported the increase of PlGF expression in cirrhotic liver, increase in plasma PlGF levels in patients with alcoholic hepatitis and a linear correlation between plasma PlGF levels and HVPG. Our findings support this evidence as we have also observed increased plasma PlGF levels in patients with hepatitis C virus and alcohol-induced liver cirrhosis as well as linear correlation between plasma PlGF levels and HVPG. PlGF levels at the hepatic vein did not differ from the levels at the peripheral vein, suggesting that PlGF levels remain stable after

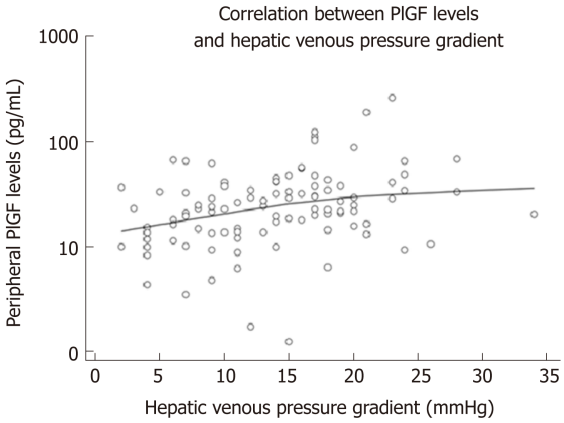


Figure 3 Relationship between peripheral placental growth factor levels and hepatic venous pressure gradient. PlGF: Placental growth factor.

passing to the systemic circulation. The reason why in our study hepatic PlGF levels did not correlate to HVPG needs further research and we are planning to explore this phenomenon with a higher study sample. Furthermore, in our study we have observed that plasma PlGF levels were predictors of CSPH (sensitivity 55.7%, specificity 76.7%) and SPH (sensitivity 59%, specificity 79%) in patients with cirrhosis. Another important finding of our study was the association of plasma PlGF levels with high-risk esophageal varices. The cut-off value of 19.83 pg/mL predicted high-risk esophageal varices with high sensitivity 90% and high negative predictive value 94.4%. Hence plasma PlGF levels could be promising candidates for noninvasive diagnosis of PH and help distinguish the patients with high risk for variceal bleeding.

Nogo-A is a member of reticulon 4 (RTN4) family of membrane-associated multifunctional proteins[40]. There are three isoforms of RTN4 protein: Nogo-A, Nogo-B and Nogo C. Nogo-A was initially most widely studied as a neurite growth inhibitor and was believed to be mainly expressed in the nervous system[36,41]. Apart from the nervous system, Nogo-A was shown to be expressed in the heart tissue, testis and liver, although in the latter the signal was considered unspecific[42]. Furthermore, a recent study by Ramo *et al*[43] has reported that Nogo-A and Nogo-B are simultaneously expressed in human hepatoma, fibroblast and neuronal cells, concluding than none of the isoforms should be considered a cell type specific isoform and revealed a wider range of functions for Nogo-A outside the nervous system. Another study addressing the expression of Nogo-A in the liver was performed by Hao *et al*[24], where the authors reported that Nogo-A was highly expressed in four liver cancer cell lines *in vitro* and the depletion of Nogo-A protein suppressed cancer cell proliferation. To our best knowledge, this study is the first to demonstrate the relationship of Nogo-A with liver cirrhosis and PH in humans. Furthermore, the cut-off value of 1.12 ng/mL helped to differentiate CSPH with high specificity (93.1%) and positive predictive value (92.9%), showing that Nogo-A might be used as a non-invasive marker for the diagnosis of CSPH. Nogo-A levels at the hepatic vein were significantly higher than in the peripheral vein, suggesting that the protein undergoes some metabolism processes in the systemic circulation. However to explain this phenomenon further mechanistic studies are required.

In conclusion, plasma PlGF levels were higher and Nogo-A plasma levels were lower in patients with liver cirrhosis and PH. Both biomarkers showed correlation with HVPG and only moderate predictive value in determining clinically significant, severe portal hypertension and high-risk esophageal varices.

The main limitation of our study is the lack of mechanistic studies concerning Nogo-A protein in healthy liver tissue and cirrhotic tissue, which could better explain the expression of the protein in healthy and diseased liver.

Table 2 Diagnostic performance of placental growth factor and Nogo-A for clinically significant, severe portal hypertension and high-risk varicose veins

	Cut-off value[1]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P* value
CSPH						
PLGF	25 pg/mL	55.7	76.7	84.8	42.6	0.003
Nogo-A	1.12 ng/mL	36.6	93.1	92.9	37.5	0.01
SPH						
PlGF	26.8 pg/mL	59.0	79.5	81.8	55.4	0.0001
Nogo-A	1 ng/mL	33.3	92.5	87	48	0.014
High-risk varicose veins						
PlGF	19.83 pg/mL	90.5	44.2	30.6	94.4	0.034

[1]Cut-off values with most accurate sensitivity and specificity to differentiate patients with the condition from patients without the condition. PlGF: Placental growth factor; SPH: Severe portal hypertension; CSPH: Clinically significant portal hypertension; PPV: Positive predictive value; NPV: Negative predictive value.

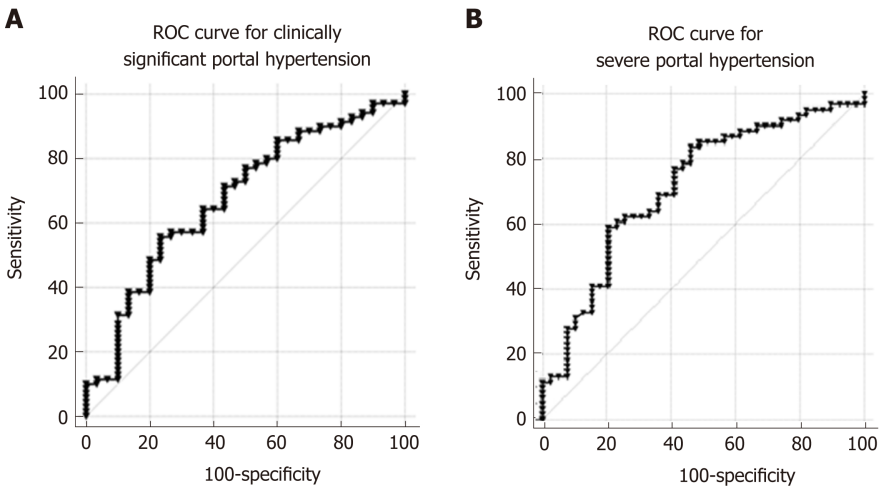


Figure 4 Receiver operating characteristic curves showing placental growth factor prediction of clinically significant portal hypertension (A) and severe portal hypertension (B). ROC: Receiver operating characteristic.

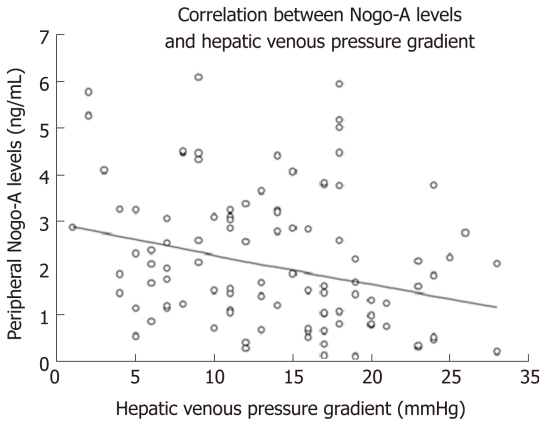


Figure 5 Relationship between peripheral Nogo-A levels and hepatic venous pressure gradient.

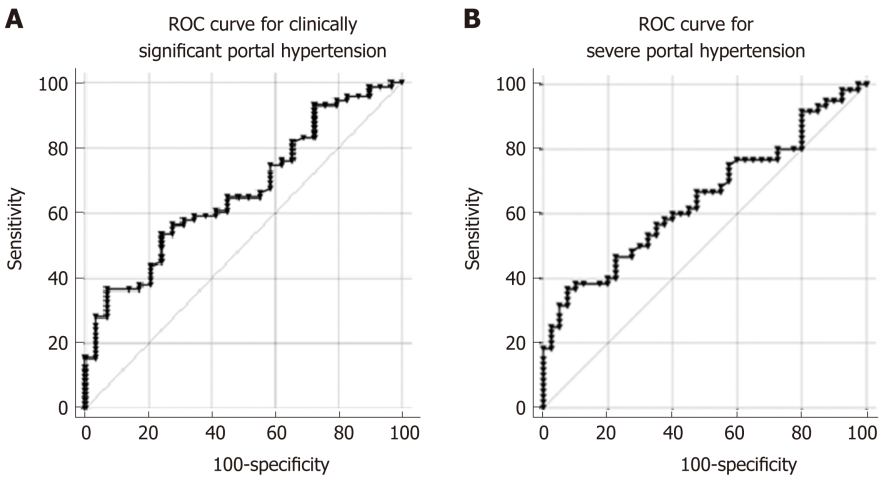


Figure 6 Receiver operating characteristic curves showing Nogo-A protein prediction of clinically significant portal hypertension (A) and severe portal hypertension (B). ROC: Receiver operating characteristic.

ARTICLE HIGHLIGHTS

Research background

Portal hypertension (PH) is a consequence of liver cirrhosis and can cause serious life-threatening complications. The degree of portal hypertension is one of the most important prognostic factors and is defined by the hepatic venous pressure gradient (HVPG). In order to optimize the care of patients with liver cirrhosis, it is essential to detect early PH and prevent the development of clinically significant PH (CSPH) to avoid decompensation. As current options for prevention and treatment of CSPH are limited to attenuating splanchnic vasodilatation, new insights and deeper knowledge about pathogenetic mechanisms of PH and molecules involved, would aid in search for newer and more effective treatment strategies.

Research motivation

With growing burden on chronic liver diseases worldwide, the optimization of care provided to the patients with liver cirrhosis has become a priority. According to the Baveno VI consensus meeting the detection and treatment of early PH as well as the prevention and timely treatment of CSPH, in order to avoid complications and decompensation, are of utmost importance. The consensus has encouraged the search for new treatment strategies to target different patho-genetic components of PH. Recent studies suggest that one of these alternative pathogenetic components is pathologic angiogenesis. Pathologic angiogenesis has been reported to result from the upregulation of proangiogenic factors and simultaneous downregulation of angiogenesis inhibitors, thus both stimuli might be important and contribute to the pa-thophysiological mechanisms. This prospective study was aimed to gain deeper knowledge on the molecules involved in abnormal angiogenesis-one of the pa-thogenetic components of PH. PlGF has previously been associated with portal hypertension in animal models; however, data in patients with liver cirrhosis are scarce. Nogo-A protein has not been previously evaluated in patients with liver cirrhosis and PH.

Research objectives

In this study we aimed to evaluate plasma levels of PlGF and Nogo-A in liver cirrhosis patients with normal portal pressure and portal hypertension as well as in controls. We also aimed to evaluate the potential of plasma PlGF and Nogo-A levels as biomarkers to predict CSPH and SPH as well as complications of portal hypertension.

Research methods

A cohort of 122 patients with hepatitis C virus and/or alcohol-induced liver cirrhosis with characterized HVPG were included in the study. Demographic data, medical history, Child-Turcotte-Pugh and Model of End Stage liver disease score, clinical chemistry, liver stiffness values were recorded on the day of the procedure prior HVPG measurement. The degree of portal hypertension was determined by the invasive HVPG measurement. Nogo-A and placental growth factor levels in plasma were evaluated using enzyme liked im40munosorbent assay. The control group consisted of 30 healthy age- and sex- matched individuals

Research results

We have demonstrated differences of proangiogenic mediator PlGF and angiogenesis inhibitor Nogo-A plasma levels in patients with liver cirrhosis, when compared to controls. We have also determined a clear correlation of these mediators to PH and complications of PH. Furthermore, we have for the first time demonstrated that plasma concentration of Nogo-A is lower in liver cirrhosis and PH, as well as the predictive values of plasma PlGF and Nogo-A levels in

diagnosing CSPH, SPH and PH complications. Futher research will be addressed to evaluate Nogo-A expression in healthy and cirrhotic liver.

Research conclusions

We have for the first time demonstrated that plasma concentration of Nogo-A is lower in liver cirrhosis and PH, as well as the predictive values of plasma PlGF and Nogo-A levels in diagnosing CSPH, SPH and PH complications. We believe that our study expands the knowledge on pathologic angiogenesis and its role in the pathogenesis of PH as well as molecules involved. We have demonstrated that indeed PH is a complicated pathology with multiple pathogenetic pathways, which are important in optimizing the care of patients with portal hypertension. This information provides further insights in the pathogenesis of PH in liver cirrhosis and might be useful in creating new noninvasive diagnostic models for CSPH, as it is one of the most important prognostic factors for patients with liver cirrhosis. We have proposed the new hypothesis that Nogo-A protein is associated with portal hypertension and pathologic angiogenesis. This study examined two novel non-invasive markers of portal hypertension, which could be useful in creatinf noninvasive diagnostic models for PH, or new treatment targets. We have demonstrated that two biomarkers of pathologic angiogenesis have moderate predictive value in diagnosing CSPH and SPH as well as high risk esophageal varices. Our study demonstrated that biomarkers of pathologic angiogenesis are associated with liver cirrhosis and PH and have moderate ability to predict CSPH and SPH. These findings might be useful in creating new noninvasive diagnostic models for PH as well as new treatment targets of PH.

Research perspectives

Future research will be directed towards gaining more detailed information about Nogo-A protein expression in healthy and cirrhotic liver as well as further understanding of Nogo-A protein roles outside the central nervous system. We plan to conduct mechanistic studies, using liver cell models as well as liver tissue biopsy specimens.

REFERENCES

1 **Snowdon VK**, Guha N, Fallowfield JA. Noninvasive evaluation of portal hypertension: emerging tools and techniques. *Int J Hepatol* 2012; **2012**: 691089 [PMID: 22720166 DOI: 10.1155/2012/691089]
2 **Berzigotti A**. Non-invasive evaluation of portal hypertension using ultrasound elastography. *J Hepatol* 2017; **67**: 399-411 [PMID: 28223101 DOI: 10.1016/j.jhep.2017.02.003]
3 **Stefanescu H**, Procopet B. Noninvasive assessment of portal hypertension in cirrhosis: liver stiffness and beyond. *World J Gastroenterol* 2014; **20**: 16811-16819 [PMID: 25492995 DOI: 10.3748/wjg.v20.i45.16811]
4 **de Franchis R**; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]
5 **Schwabl P**, Laleman W. Novel treatment options for portal hypertension. *Gastroenterol Rep (Oxf)* 2017; **5**: 90-103 [PMID: 28533907 DOI: 10.1093/gastro/gox011]
6 **Garbuzenko DV**, Arefyev NO, Kazachkov EL. Antiangiogenic therapy for portal hypertension in liver cirrhosis: Current progress and perspectives. *World J Gastroenterol* 2018; **24**: 3738-3748 [PMID: 30197479 DOI: 10.3748/wjg.v24.i33.3738]
7 **Bosch J**, Abraldes JG, Fernández M, García-Pagán JC. Hepatic endothelial dysfunction and abnormal angiogenesis: new targets in the treatment of portal hypertension. *J Hepatol* 2010; **53**: 558-567 [PMID: 20561700 DOI: 10.1016/j.jhep.2010.03.021]
8 **Bocca C**, Novo E, Miglietta A, Parola M. Angiogenesis and Fibrogenesis in Chronic Liver Diseases. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 477-488 [PMID: 28210697 DOI: 10.1016/j.jcmgh.2015.06.011]
9 **Geerts AM**, De Vriese AS, Vanheule E, Van Vlierberghe H, Mortier S, Cheung KJ, Demetter P, Lameire N, De Vos M, Colle I. Increased angiogenesis and permeability in the mesenteric microvasculature of rats with cirrhosis and portal hypertension: an in vivo study. *Liver Int* 2006; **26**: 889-898 [PMID: 16911473 DOI: 10.1111/j.1478-3231.2006.01308.x]
10 **Mejias M**, Garcia-Pras E, Tiani C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 2009; **49**: 1245-1256 [PMID: 19137587 DOI: 10.1002/hep.22758]
11 **Fernandez M**, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology* 2004; **126**: 886-894 [PMID: 14988842 DOI: 10.1053/j.gastro.2003.12.012]
12 **Fernandez M**, Mejias M, Angermayr B, Garcia-Pagan JC, Rodés J, Bosch J. Inhibition of VEGF receptor-2 decreases the development of hyperdynamic splanchnic circulation and portal-systemic collateral vessels in portal hypertensive rats. *J Hepatol* 2005; **43**: 98-103 [PMID: 15893841 DOI: 10.1016/j.jhep.2005.02.022]
13 State-of-the-art conference on azidothymidine therapy for early HIV infection. *Am J Med* 1990; **89**: 335-344 [PMID: 2203263 DOI: 10.1111/j.1365-2036.2011.04896.x]
14 **Coriat R**, Gouya H, Mir O, Ropert S, Vignaux O, Chaussade S, Sogni P, Pol S, Blanchet B, Legmann P, Goldwasser F. Reversible decrease of portal venous flow in cirrhotic patients: a positive side effect of sorafenib. *PLoS One* 2011; **6**: e16978 [PMID: 21340026 DOI: 10.1371/journal.pone.0016978]
15 **Fernández M**, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol* 2009; **50**: 604-620 [PMID: 19157625 DOI: 10.1016/j.jhep.2008.12.011]
16 **Dewerchin M**, Carmeliet P. PlGF: a multitasking cytokine with disease-restricted activity. *Cold Spring Harb Perspect Med* 2012; **2** [PMID: 22908198 DOI: 10.1101/cshperspect.a011056]
17 **Van Steenkiste C**, Ribera J, Geerts A, Pauta M, Tugues S, Casteleyn C, Libbrecht L, Olievier K, Schroyen B, Reynaert H, van Grunsven LA, Blomme B, Coulon S, Heindryckx F, De Vos M, Stassen JM, Vinckier S, Altamirano J, Bataller R, Carmeliet P, Van Vlierberghe H, Colle I, Morales-Ruiz M. Inhibition of placental growth factor activity reduces the severity of fibrosis, inflammation, and portal hypertension in

cirrhotic mice. *Hepatology* 2011; **53**: 1629-1640 [PMID: 21520176 DOI: 10.1002/hep.24238]
18 **Li X**, Yao QY, Liu HC, Jin QW, Xu BL, Zhang SC, Tu CT. Placental growth factor silencing ameliorates liver fibrosis and angiogenesis and inhibits activation of hepatic stellate cells in a murine model of chronic liver disease. *J Cell Mol Med* 2017; **21**: 2370-2385 [PMID: 28378526 DOI: 10.1111/jcmm.13158]
19 **Nyberg P**, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005; **65**: 3967-3979 [PMID: 15899784 DOI: 10.1158/0008-5472.CAN-04-2427]
20 **Gana JC**, Serrano CA, Ling SC. Angiogenesis and portal-systemic collaterals in portal hypertension. *Ann Hepatol* 2016; **15**: 303-313 [PMID: 27049484 DOI: 10.5604/16652681.1198799]
21 **Sarkey JP**, Chu M, McShane M, Bovo E, Ait Mou Y, Zima AV, de Tombe PP, Kartje GL, Martin JL. Nogo-A knockdown inhibits hypoxia/reoxygenation-induced activation of mitochondrial-dependent apoptosis in cardiomyocytes. *J Mol Cell Cardiol* 2011; **50**: 1044-1055 [PMID: 21420413 DOI: 10.1016/j.yjmcc.2011.03.004]
22 **Osborne SL**, Corcoran SL, Prinjha RK, Moore SE. Nogo A expression in the adult enteric nervous system. *Neurogastroenterol Motil* 2004; **16**: 465-474 [PMID: 15306002 DOI: 10.1111/j.1365-2982.2004.00527.x]
23 **Pernet V**. Nogo-A in the visual system development and in ocular diseases. *Biochim Biophys Acta Mol Basis Dis* 2017; **1863**: 1300-1311 [PMID: 28408340 DOI: 10.1016/j.bbadis.2017.04.008]
24 **Hao CQ**, Zhou Y, Wang JP, Peng MJ, Xie YM, Kang WZ, Sun L, Wang PZ, Wan CL, He L, Cai L, Jia ZS. Role of NogoA in the regulation of hepatocellular carcinoma SMMC7721 cell apoptosis. *Mol Med Rep* 2014; **9**: 1743-1748 [PMID: 24626842 DOI: 10.3892/mmr.2014.2050]
25 **Tata M**, Ruhrberg C, Fantin A. Vascularisation of the central nervous system. *Mech Dev* 2015; **138** Pt 1: 26-36 [PMID: 26222953 DOI: 10.1016/j.mod.2015.07.001]
26 **Wälchli T**, Pernet V, Weinmann O, Shiu JY, Guzik-Kornacka A, Decrey G, Yüksel D, Schneider H, Vogel J, Ingber DE, Vogel V, Frei K, Schwab ME. Nogo-A is a negative regulator of CNS angiogenesis. *Proc Natl Acad Sci USA* 2013; **110**: E1943-E1952 [PMID: 23625008 DOI: 10.1073/pnas.1216203110]
27 **Zhang D**, Utsumi T, Huang HC, Gao L, Sangwung P, Chung C, Shibao K, Okamoto K, Yamaguchi K, Groszmann RJ, Jozsef L, Hao Z, Sessa WC, Iwakiri Y. Reticulon 4B (Nogo-B) is a novel regulator of hepatic fibrosis. *Hepatology* 2011; **53**: 1306-1315 [PMID: 21480333 DOI: 10.1038/jid.2014.371]
28 **Men R**, Wen M, Dan X, Zhu Y, Wang W, Li J, Wu W, Liu X, Yang L. Nogo-B: A potential indicator for hepatic cirrhosis and regulator in hepatic stellate cell activation. *Hepatol Res* 2015; **45**: 113-122 [PMID: 24606149 DOI: 10.1111/hepr.12324]
29 **Zhao B**, Chun C, Liu Z, Horswill MA, Pramanik K, Wilkinson GA, Ramchandran R, Miao RQ. Nogo-B receptor is essential for angiogenesis in zebrafish via Akt pathway. *Blood* 2010; **116**: 5423-5433 [PMID: 20813898 DOI: 10.1182/blood-2010-02-271577]
30 **Teng RJ**, Rana U, Afolayan AJ, Zhao B, Miao QR, Konduri GG. Nogo-B receptor modulates angiogenesis response of pulmonary artery endothelial cells through eNOS coupling. *Am J Respir Cell Mol Biol* 2014; **51**: 169-177 [PMID: 24568601 DOI: 10.1165/rcmb.2013-0298OC]
31 **Yu J**, Fernández-Hernando C, Suarez Y, Schleicher M, Hao Z, Wright PL, DiLorenzo A, Kyriakides TR, Sessa WC. Reticulon 4B (Nogo-B) is necessary for macrophage infiltration and tissue repair. *Proc Natl Acad Sci USA* 2009; **106**: 17511-17516 [PMID: 19805174 DOI: 10.1073/pnas.0907359106]
32 **Groszmann RJ**, Wongcharatrawee S. The hepatic venous pressure gradient: anything worth doing should be done right. *Hepatology* 2004; **39**: 280-282 [PMID: 14767976 DOI: 10.1002/hep.20062]
33 **Marcellin P**, Kutala BK. Liver diseases: A major, neglected global public health problem requiring urgent actions and large-scale screening. *Liver Int* 2018; **38** Suppl 1: 2-6 [PMID: 29427496 DOI: 10.1111/liv.13682]
34 **Sieber CC**, Sumanovski LT, Stumm M, van der Kooij M, Battegay E. In vivo angiogenesis in normal and portal hypertensive rats: role of basic fibroblast growth factor and nitric oxide. *J Hepatol* 2001; **34**: 644-650 [PMID: 11434609 DOI: 10.1016/S0168-8278(00)00064-7]
35 **Cejudo-Martín P**, Ros J, Navasa M, Fernández J, Fernández-Varo G, Ruiz-del-Arbol L, Rivera F, Arroyo V, Rodés J, Jiménez W. Increased production of vascular endothelial growth factor in peritoneal macrophages of cirrhotic patients with spontaneous bacterial peritonitis. *Hepatology* 2001; **34**: 487-493 [PMID: 11526533 DOI: 10.1053/jhep.2001.27093]
36 **Salcedo Mora X**, Sanz-Cameno P, Medina J, Martín-Vílchez S, García-Buey L, Borque MJ, Moreno-Otero R. Association between angiogenesis soluble factors and disease progression markers in chronic hepatitis C patients. *Rev Esp Enferm Dig* 2005; **97**: 699-706 [PMID: 16351462]
37 **Krawczyk M**, Zimmermann S, Hess G, Holz R, Dauer M, Raedle J, Lammert F, Grünhage F. Panel of three novel serum markers predicts liver stiffness and fibrosis stages in patients with chronic liver disease. *PLoS One* 2017; **12**: e0173506 [PMID: 28301573 DOI: 10.1371/journal.pone.0173506]
38 **Li X**, Jin Q, Yao Q, Zhou Y, Zou Y, Li Z, Zhang S, Tu C. Placental Growth Factor Contributes to Liver Inflammation, Angiogenesis, Fibrosis in Mice by Promoting Hepatic Macrophage Recruitment and Activation. *Front Immunol* 2017; **8**: 801 [PMID: 28744285 DOI: 10.3389/fimmu.2017.00801]
39 **Van Steenkiste C**, Geerts A, Vanheule E, Van Vlierberghe H, De Vos F, Olievier K, Casteleyn C, Laukens D, De Vos M, Stassen JM, Carmeliet P, Colle I. Role of placental growth factor in mesenteric neoangiogenesis in a mouse model of portal hypertension. *Gastroenterology* 2009; **137**: 2112-24.e1-6 [PMID: 19751735 DOI: 10.1053/j.gastro.2009.08.068]
40 **Yang YS**, Strittmatter SM. The reticulons: a family of proteins with diverse functions. *Genome Biol* 2007; **8**: 234 [PMID: 18177508 DOI: 10.1186/gb-2007-8-12-234]
41 **Sui YP**, Zhang XX, Lu JL, Sui F. New Insights into the Roles of Nogo-A in CNS Biology and Diseases. *Neurochem Res* 2015; **40**: 1767-1785 [PMID: 26266872 DOI: 10.1007/s11064-015-1671-5]
42 **Huber AB**, Weinmann O, Brösamle C, Oertle T, Schwab ME. Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. *J Neurosci* 2002; **22**: 3553-3567 [PMID: 11978832 DOI: 10.1523/JNEUROSCI.22-09-03553.2002]
43 **Rämö O**, Kumar D, Gucciardo E, Joensuu M, Saarekas M, Vihinen H, Belevich I, Smolander OP, Qian K, Auvinen P, Jokitalo E. NOGO-A/RTN4A and NOGO-B/RTN4B are simultaneously expressed in epithelial, fibroblast and neuronal cells and maintain ER morphology. *Sci Rep* 2016; **6**: 35969 [PMID: 27786289 DOI: 10.1038/srep35969]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2947-2960

DOI: 10.3748/wjg.v25.i23.2947

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

SYSTEMATIC REVIEWS

Expression of genes that control core fucosylation in hepatocellular carcinoma: Systematic review

Pamela A Norton, Anand S Mehta

ORCID number: Pamela A Norton (0000-0003-1327-9250); Anand S Mehta (0000-0002-9846-9389).

Author contributions: Norton PA and Mehta AS designed the research; Norton PA performed the searches; Norton PA and Mehta AS analyzed the data and wrote the paper.

Conflict-of-interest statement: The authors declare no conflicts.

PRISMA 2009 Checklist statement: This systematic review was conducted according to the PRISMA 2009 Checklist (http://www.prisma-statement.org/).

Manuscript source: Invited manuscript

Received: March 14, 2019
Peer-review started: March 14, 2019
First decision: April 11, 2019
Revised: April 25, 2019
Accepted: May 18, 2019
Article in press: May 18, 2019

Pamela A Norton, Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA 19102, United States

Anand S Mehta, Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, SC 29425, United States

Corresponding author: Pamela A Norton, PhD, Associate Professor, Department of Microbiology and Immunology, Drexel University College of Medicine, 245 N. 15th Street, Philadelphia, PA 19102, United States. pan29@drexel.edu
Telephone: +1-215-762-4146

Abstract

BACKGROUND

Changes in N-linked glycosylation have been observed in the circulation of individuals with hepatocellular carcinoma. In particular, an elevation in the level of core fucosylation has been observed. However, the mechanisms through which core fucose is increased are not well understood. We hypothesized that a review of the literature and related bioinformatic review regarding six genes known to be involved in the attachment of core fucosylation, the synthesis of the fucosylation substrate guanosine diphosphate (GDP)-fucose, or the transport of the substrate into the Golgi might offer mechanistic insight into the regulation of core fucose levels.

AIM

To survey the literature to capture the involvement of genes regulating core N-linked fucosylation in hepatocellular carcinoma

METHODS

The PubMed biomedical literature database was searched for the association of hepatocellular carcinoma and each of the core fucose-related genes and their protein products. We also queried The Cancer Genome Atlas Liver hepatocellular carcinoma (LIHC) dataset for genetic, epigenetic and gene expression changes for the set of six genes using the tools at cBioportal.

RESULTS

A total of 27 citations involving one or more of the core fucosylation-related genes (FPGT, FUK, FUT8, GMDS, SLC35C1, TSTA3) and hepatocellular carcinoma were identified. The same set of gene symbols was used to query the 371 patients with liver cancer in the LIHC dataset to identify the frequency of mRNA over or under expression, as well as non-synonymous mutations, copy number variation and methylation level. Although all six genes trended to more

Published online: June 21, 2019

P-Reviewer: Jin C, Li W, Wang L, Zhang WC
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

samples displaying over expression relative to under-expression, it was noted that a number of tumor samples had undergone amplification of the genes of the *de novo* synthesis pathway, GMDS (27 samples) and TSTA3 (78 samples). In contrast, the other four genes had undergone amplification in 2 or fewer samples.

CONCLUSION
Amplification of genes involved in the *de novo* pathway for generation of GDP-fucose, GMDS and TSTA3, likely contributes to the elevated core fucose observed in hepatocellular carcinoma.

Key words: Liver cancer; N-linked glycosylation; Fucose; Guanosine diphosphate fucose; Hepatocellular carcinoma

Core tip: The increased level of core fucosylated N-linked glycoproteins detected in some patients with hepatocellular carcinoma may occur *via* several potential molecular mechanisms. We report that the genes GMDS and TSTA3, which are associated with the *de novo* synthesis of the fucose donor species, guanosine diphosphate fucose, can be amplified in some hepatocellular carcinoma patients. Amplification seems to be the most common genetic alteration affecting the genes that control the production of core fucosylated proteins.

Citation: Norton PA, Mehta AS. Expression of genes that control core fucosylation in hepatocellular carcinoma: Systematic review. *World J Gastroenterol* 2019; 25(23): 2947-2960
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2947.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2947

INTRODUCTION

Changes in N-linked glycosylation of liver-derived serum proteins have been associated with the development of hepatocellular carcinoma (HCC)[1-7]. Of particular note, an increase in alpha1,6-linked core fucose modified serum proteins, both in total as well as on individual proteins has been reported in some patients with HCC by us and others[6,8-12]. However, there is considerable variation, with not all individuals showing the same magnitude of glycosylation change. An understanding of the sources of variability in core fucosylation might allow the use of such changes as potential biomarkers for early, non-invasive detection of HCC.

In humans and other mammals, there is only a single alpha-(1,6)-fucosyltransferase (also called alpha1-6 FucT); that enzyme attaches fucose *via* 1,6 linkage to the innermost core N-acetylglucosamine[5,13,14]. This enzyme is the product of the FUT8 gene. Increases in FUT8 gene expression have been associated with HCC and liver steatosis[15-17]. Loss of the FUT8 gene in mice is associated with reduced tumor formation along with impaired liver regeneration[18]. However, core fucose levels can also be affected by the level of the guanosine diphosphate (GDP)-fucose substrate and/or its transport into the Golgi[5,13,19,20].

GDP-fucose is generated from L-fucose by two distinct pathways (Figure 1), *de novo* synthesis and salvage[5,13]. In *de novo* synthesis, GDP-mannose is converted to GDP-4-keto-6-deoxymannose by the enzyme GDP-mannose 4,6 dehydratase (GMDS). This intermediate is converted to GDP-fucose by the bifunctional GDP-L-fucose synthetase 1 (FX)[21], encoded by the TSTA3 gene, which performs both final synthetic steps. Alternatively, in the salvage pathway, L-fucose kinase (encoded by the gene FUK) phosphorylates L-fucose, which is then acted upon by fucose-1-phosphate guanylyltransferase (encoded by the gene FPGT) to produce GDP-fucose[5,13,14]. These reactions all occur in the cytoplasm. GDP-fucose enters the Golgi complex, where the FUT8 enzyme resides, *via* the membrane anchored GDP-fucose transporter 1 (encoded by gene SLC35C1; also called FUCT1). Thus, in principle, alteration of expression of the genes encoding any of these enzymes could affect the level of core fucosylated proteins secreted from HCC cells into the circulation.

In this review, we describe the results of a search of the literature for reports linking

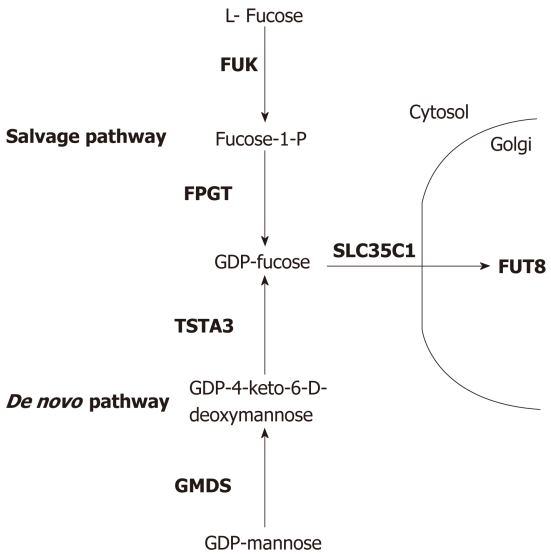


Figure 1 Diagram of the proteins and precursor/product sugars involved with guanosine diphosphate-fucose synthesis, Golgi transport and attachment of core fucose to N-linked glycoproteins. Proteins are designated by the symbols of the genes that encode them (bold). The more dominant *de novo* synthesis pathway is shown at the bottom, the salvage pathway is at the top. Diagram is based on previous studies[5,13]. GDP: Guanosine diphosphate.

any of the above mentioned proteins or genes with HCC. We also investigate possible genetic mechanisms that could produce heterogeneity in the levels of alpha 1,6-linked core fucose in liver-derived serum glycoproteins. The genes associated with core fucosylation were evaluated for changes in copy number, methylation, mutation status and mRNA expression level in the data set generated by the Cancer Genome Atlas project, which included 373 HCC samples. This analysis has revealed several potential mechanisms that could account for increased core fucose in the context of HCC.

MATERIALS AND METHODS

This systematic review was conducted according to PRISMA guidelines (http://www.prisma-statement.org/). We searched PubMed in January of 2019 for the combination of "hepatocellular carcinoma" along with each individual HUGO gene symbols or protein name (Table 1). We also recorded the number of citations provided for each gene in the Uniprot database on the same date (https://www.uniprot.org/).

The molecular analyses described here use data generated by the TCGA Research Network: http://cancergenome.nih.gov/. The Cancer Genome Atlas Project has produced a large amount of data to detect somatic DNA mutations, DNA copy number variations, DNA methylation alterations and changes in mRNA levels in 20 cancer types[22]. The precise amount of data, in terms of the numbers of patient samples measured as well as the type of data available, including access to other clinical parameters, varies with the specific set of tumor tissues. However, the amount of data provides a rich resource for evaluating genetic changes. The HCC dataset (Liver hepatocellular carcinoma, LIHC) used for the analyses in this report were captured in May, 2016, prior to official publication[23]. At that time, the database included 373 samples with mRNA data (RSEM normalized HiSeqRNASeq V2). These represent samples collected from 371 individual patients. Copy number variation data was based on 370 cases analyzed by Affymetrix SNP6. Methylation data was analyzed using Illumina HumanMethylation450 bead chip. Since this data set was unpublished at the time and designated as Provisional by TCGA, mutation data might not be complete.

Resources used and analyses performed

Identification of genetic and epigenetic changes as well as changes in gene expression was carried out on the LIHC dataset using the tools at the cBioportal website (http://www.cbioportal.org/)[24,25]. The ID symbols for the six genes of interest for core fucose production are FPGT, FUT8, FUK, GMDS, SLC35C1 and TSTA3. These were searched individually and as a set for the presence and types of mutations, or for

Table 1 Literature citations to core fucosylation related genes and hepatocellular carcinoma

Gene symbol	Citations (gene)	Citations (protein name)	EC[2]	Uniprot ID	Uniprot citations
FPGT	0	0	2.7.7.30	O14772	7
FUK	0	0	2.7.1.52	Q8NOW3	4
FUT8	16	17	2.4.1.68	Q9BYC5	15
GMDS	2	4[1]	4.2.1.47	O60547	25
SLC35C1	1	1	NA	Q96A29	13
TSTA3	4	13	1.1.1.271	Q13630	12

[1]The protein name searched was mannose dehydratase;
[2]Enzyme commission; NA: Not applicable.

changes in copy number. The cBioportal Oncoprint tool was used to provide a graphic summary of major genetic alterations and changes in gene expression. Increases or decreases in mRNA level were based on a Z-score threshold of 2.0 or more standard deviations from the mean of the reference population. The reference population was the samples that are scored diploid for each gene.

Correlations between overall mRNA levels in the sample set were analyzed by comparison with gene copy number, either the relative linear copy number (from Affymetrix SNP6) or the GISTIC2.0 (Genomic Identification of Significant Targets in Cancer) algorithm calls[26]. DNA methylation levels were measured with the Illumina Infinium HM450 assay, expressed as beta values (ratio of methylated and unmethylated alleles) and compared with gene expression level. In the event of multiple methylation probes per gene, the most negatively correlated with expression is shown. The cBioportal Co-Expression tool was used to view the genes across the samples with an expression pattern that correlated most positively or negatively with the query gene and to extract the cytogenetic location (cytoband) of all genes.

RESULTS

Literature search strategy and results

Searching the PubMed database for "hepatocellular carcinoma" along with "core fucose" yielded 31 references, and "hepatocellular carcinoma" along with "fucose" produced 153. Searching PubMed revealed only a limited number of citations that included both "hepatocellular carcinoma" and either the gene symbol or protein name for each of the six genes encoding enzymes involved in attaching core fucose (FUT8), generating GDP-fucose (GMDS and TSTA3 *via* the de novo pathway, FUK and FPGT *via* the salvage pathway), or the anti-porter involved in transferring that substrate into the Golgi (SLC35C1). For comparison, the UniProt IDs (https://www.uniprot.org/) for each gene product and the number of references listed there also are shown in Table 1. The enzyme commission number is provided, where pertinent.

The combination of "hepatocellular carcinoma" and "FUT8" had the most citations with 16[15,18,27-40], with 17 references found using "fucosyltransferase 8" instead. The two sets were mostly overlapping, but some of the latter referred only to other fucosyltransferases, and thus were omitted, leaving only three additional relevant citations[41-43]. Substituting the TSTA3 gene produced four hits[21,44-46], whereas searching for the gene's protein product, FX, returned 13 citations. However, the titles or abstracts of the latter revealed that several used "FX" to refer to fucoxanthin, clotting factor X or the radiologic term "Gy/fx". Eliminating these left only four references to the TSTA3 gene product[19-21,44], two of which were also produced by searching for the gene symbol. Substituting GMDS produced only two citations[19,35,47,48], only one of which was not covered in a previous search. Searching for "hepatocellular carcinoma" and "mannose dehydratase" produced four citations. Searching for SLC35C1 produced a single citation[19]. The gene symbols FPGT and FUK produced no citations when paired with "hepatocellular carcinoma", nor did the names of the protein products. Thus, removing duplicates that appeared in more than one search, we arrived at a curated set of 27 citations involving one or more of the core fucosylation-related genes and HCC.

Bioinformatic search and results

As a complement to the literature searches, we were interested in whether genetic changes to these core fucose-related genes occur in liver cancer. Using the cBioPortal

interface, the LIHC data set was queried for each of the six genes encoding enzymes involved in attaching core fucose (FUT8), generating GDP-fucose (GMDS, TSTA3, FUK, and FPGT), or the anti-porter involved in transferring that substrate into the Golgi (SLC35C1). None have been causally implicated in cancer, according to the Cancer Census (http://cancer.sanger.ac.uk/census). Using the cBioPortal's Oncoprint tool, genome-based changes in these six genes and their expression levels were compared for 370 patients with liver cancer (Figure 2 and summarized in Table 2). This display provides both the number and nature of the alterations, as well as whether these are co-occurring in the same individual patients, illustrated by examining the vertically aligned segments. None of the total changes, when examine for either single genes or for the panel of six, showed a significant correlation with overall or disease-free survival (not shown). Of particular note, the trend for all genes is toward mRNA increase versus decrease (Figure 2 and Table 2). The behavior of each gene will be considered in the context of the role of its gene product in producing core fucose.

De novo pathway genes

The trend toward increased gene expression in HCC is most striking for GMDS (27 samples) and TSTA3 (78 samples), with both gene products involved in the *de novo* pathway for fucose synthesis. Neither gene underwent non-synonymous or truncating mutations that would change the encoded protein. However, both genes were scored as amplified in multiple samples: 23 for GMDS and 58 for TSTA3. Only three samples had both amplifications. Closer examination of the GISTIC2 calls indicated a strong bias for both genes to experience copy number gain versus loss (Figure 3). TSTA3 showed low HM450 beta values (all < 0.01), indicating a low level of methylation, which correlated negatively with mRNA levels (Spearman, r = -0.425) In contrast, GMDS showed more variable methylation levels (0.3-1.0), which were negatively correlated with mRNA level (Spearman, r = -0.363).

We queried the LIHC dataset using the cBioportal Co-expression tool to identify genes whose expression pattern across the patient set correlated with that of either GMDS or TSTA3, with the goal of better understanding the basis for the amplifications. The top 10 ranked genes with similar expression patterns are listed in Tables 3 and 4. We see a tendency for the top co-expressed genes to be located near to the GMDS and TSTA3 genomic loci, which lie at chromosomal intervals 6p25 and 8q24.3, respectively. Chromosomal rearrangements resulting in copy number gain are likely occurring in those vicinities.

Salvage pathway genes

FUK and FPGT form the salvage pathway for fucose synthesis, which in general contributes less GDP-fucose than the *de novo* pathway. The FUK gene was altered in 5% of patients, with two amplifications, mRNA upregulation in 13 and downregulation in 3 (Figure 2, Table 2). No mutations that would affect the encoded protein were detected. There was a trend toward shallow deletion (single copy loss) versus gain (Figure 4). In general, the gene appears to be highly methylated, with almost all beta-values falling between 0.9-1.0. The FPGT gene was altered in 7% of samples, with 15 upregulated and 6 downregulated (Figure 2, Table 2). There were two distinct non-synonymous point mutations, one amplification and one deep deletion. As with FUK, there was a trend toward shallow deletions versus gains (Figure 4). HM450 beta-values were almost all < 0.10, indicating little methylation.

The ten genes whose expression correlated most highly with FUK and FPGT are shown in Tables 5 and 6, respectively. The FUK gene is located at chromosome 16q22.1, and all ten genes also map to 16q, with the majority mapping to 16q22.1. The FUK gene shows 152 samples with shallow deletion/hemizygosity (Figure 4), and only 25 samples exhibiting an increase in copy number, with the majority of 187 being called as diploid. Table 6 shows the ten genes that correlate most highly with FPGT; FPGT maps to chromosome 1p31.1, and the majority of the ten genes map to 1p31. Thus, for both salvage pathway genes, there is a trend toward copy number loss, despite little evidence of mRNA down-regulation.

GDP-fucose transport and transferase genes

The final two genes encode the GDP-fucose transporter SLC35C1 and the fucosyltransferase FUT8. SLC35C1 is altered in 22 cases (6%), with two amplifications, two non-synonymous mutations and 18 samples with mRNA upregulation (Figure 2 and Table 2). Methylation was strongly negatively correlated with mRNA level over a broad range of beta values ranging from 0.1 to 0.9 (Spearman, r = -0.687). This data suggests that differential DNA methylation might play a major role in determining the expression level of the SLC35C1 GDP-fucose transporter protein.

The SLC35C1 gene is located at chr. 11p11. Of the ten genes with expression pattern

Table 2 Summary of mutational and expression data for core fucose related genes

	GMDS	*TSTA3*	*FPGT*	*FUK*	*FUT8*	*SLC35C1*
Total changes (%)	13	29	7	5	6	6
mRNA up	27	78	15	13	21	18
mRNA down	0	0	6	3	0	0
Non-synonymous or truncating mutation	0	0	2	0	1	2
Copy increase	23	58	1	2	3	2
Deep deletion	3	0	1	0	2	0

most similar to SLC35C1 only one, DKFZP779M0652, maps to the same chromosomal interval (Table 7); the rest lie on different chromosomes. This predicted gene is immediately adjacent to SLC35C1 and may encode a lncRNA (ENSG00000205106). Apart from this exception, it seems unlikely that CNV plays a major role in influencing SLC35C1 expression levels. There are approximately an equal number of shallow deletions and presumably single copy gains (Figure 5).

FUT8, which encodes the enzyme directly responsible for attachment of core fucose, was found to have a low level of genetic alteration, with only 6% of samples exhibiting changes, mostly upregulation of mRNA levels, with a single exome mutation resulting in protein truncation (Figure 2 and Table 2). There were also three instances of apparent amplification and two of deep deletion. There is a trend toward shallow deletion versus low level copy number gain (Figure 5). Almost all samples exhibited low beta-values, with only 12 samples > 0.2 and the rest clustered at about 0.1, suggesting a low level of DNA methylation of FUT8 (Spearman, r = 0.232). FUT8 is at located chromosomal interval 14q23.3; none of the ten genes whose expression pattern correlated most highly with FUT8 localize to chromosome 14 (Table 8).

DISCUSSION

Search of the literature revealed little information about most of the genes involved in core fucosylation in the context of HCC (Table 1). The majority of citations recovered involved FUT8. As described in the Introduction, increased FUT8 gene expression has been observed to be increased in HCC, but not consistently, and it has been difficult to correlate mRNA expression with actual fucosylation changes occurring in tumors. The protein FX, the product of the TSTA3 gene, also has been reported as elevated in HCC, along with the proteins GMDS and FPGT, with less consistent elevation of FUT8[20]. It is thought that increased GDP-fucose substrate availability could also lead to increased core fucosylation. A previous study examined the mRNA expression levels of GMDS, FUK, FPGT, TSTA3 (FX) and SLC35C1 (GDP-Fuc Tr) in a small number of HCC samples[19]. They found a trend toward elevation of SLC35C1 and TSTA3 mRNAs in HCC versus other liver diseases, with considerable sample-to-sample variation. However, it was not possible to deduce the molecular basis for the observed expression changes.

Numerous genes have been reported to be altered in genomic analyses of liver cancers[49-51]. We sought to assess whether genome level changes to core fucose-associated genes might account for the increase of that post-translational modification that has been observed in some HCCs. Amplification of TSTA3 occurred in 58 tumors out of the 370 analyzed (16%). The tendency for amplification and thus overexpression of genes in the 8q24 chromosomal region could be driven by one or more other genes present within or near this interval, which lies at the distal terminus of chromosome 8q. Notably, MYC is present about 15 MB away at 8q24.2. MYC has been repeatedly implicated as amplified in a subset of HCCs[23,50,52,53]. Indeed, the publication describing the comprehensive analysis of the dataset used here noted that 8q exhibited frequent copy number gains[23]. Use of the Oncoprint and Plots tools at cBioPortal revealed high concordance of the GISTIC calls at TSTA3 and MYC, with 95% of the samples with TSTA3 amplification also displaying MYC amplification; 89% of MYC amplified sampled showed TSTA3 amplification. Thus, it seems reasonable to hypothesize that TSTA3 is a passenger gene, affected by known driver oncogene MYC. However, there are many other genes within this fairly large chromosomal interval.

Changes at 6p25 in the vicinity of GMDS have previously been reported in a subset of HCC cases[53,54]. Using the UCSC Genome Browser reveals that the gene FOXC1 is in very close proximity to GMDS. FOXC1 has been linked to multiple cancers, including

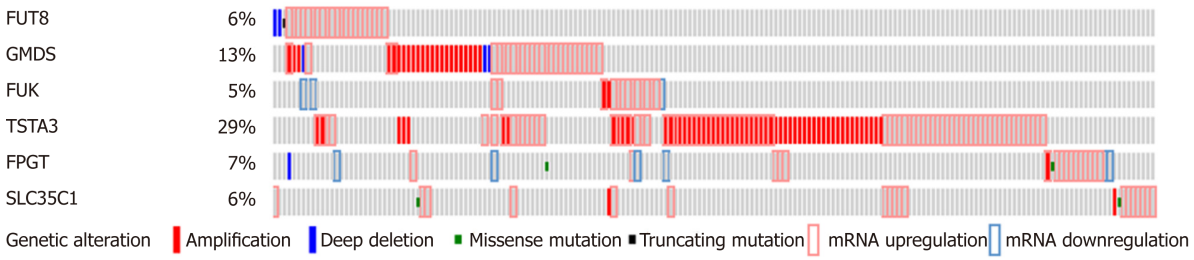


Figure 2 Oncoprint display of genetic events and gene expression changes in liver hepatocellular carcinoma samples for the six genes involved in generating core fucose. Alterations for each gene are indicated in each horizontal row, with the order of the genes in the display chosen randomly. Data for each individual patient sample is aligned vertically. Only the 185 affected samples are displayed; the other 188 showed no changes above thresholds. The types of alterations detected are indicated by the key at the bottom. Data was generated as described in the materials and methods.

HCC[55]. Elevated expression of the nearby genes SERPINB1 and SERPINB6 also has been linked with HCC[56]. Using the Oncoprint tool demonstrates that all three genes are elevated in 21 of the 23 samples with amplification of GMDS (data not shown). It is unknown whether GMDS is a simply a passenger in these amplifications, and what the specific driver(s) might be.

As to FPGT, there are no reports in the literature associating specific deletions or amplifications at 1p31.1 with HCC (based on searching PubMed for "hcc 1p31.1"), so the significance of the deletions detected at this chromosomal interval remains unclear. Abnormal methylation preceding allele loss at 16q22.1 has been associated with chronic liver disease[57]. A similar phenomenon might explain the high methylation rate at FUK, with subsequent copy number loss in a subset of the HCC samples.

Finally, neither FUT8 or SLC35C1 appear to be associated with copy number variation occurring with any frequency, with fewer than 1% of samples showing alterations, despite the elevation in expression of each gene in a subset of tumors. Changes in gene expression may relate more to transcriptional regulation, perhaps due to changing levels of transcription factors, or to mutations occurring in non-protein coding regions.

In conclusion, this was not intended as a comprehensive review of the HCC literature, but was narrowly focused on core fucose related genes in HCC. The increase in core fucosylated proteins observed in patient serum samples may be driven most often by amplification of the genes of the de novo GDP-fucose synthesis pathway, especially TSTA3. The data do not allow us to distinguish between a driver versus a passenger gene role, although the latter seems more likely. Alterations to the expression or copy number of the other core-fucose related genes likely account for a smaller subset of patients that exhibit enhanced core fucosylation. It will remain to be seen if these same trends are reflected in the relative levels of these individual proteins; it has been reported that GMDS protein levels are not well correlated with mRNA levels[19]. It is also not clear if there are actually elevated levels of GDP-fucose as a consequence of TSTA3 amplification, although it has been reported previously that elevated levels of the FX protein correlate with an increase in GDP-fucose[20]. In summary, we have identified multiple mechanisms that are likely to influence the extent of core fucosylation of the N-linked glycoproteins found in the blood of individuals with HCC.

Table 3 Genes with expression patterns that correlate positively with the *GMDS* gene

Gene symbol	Cytoband	Spearman score
FLOT1	6p21.3	0.43
SLC39A7	6p21.3	0.38
FKBPL	6p21.3	0.36
SERPINB1	6p25	0.36
PSMG4	6p25.2	0.36
IFT43	14q24.3	0.36
VARS	6p21.3	0.36
KDELR1	19q13.3	0.35
CLIC1	6p21.3	0.35
TRIM27	6p22	0.35

Table 4 Genes with expression patterns that correlate positively with the *TSTA3* gene

Gene symbol	Cytoband	Spearman score
SHARPIN	8q24.3	0.67
HGH1	8q24.3	0.68
FBXL6	8q24.3	0.68
EXOSC4	8q24.3	0.65
VPS28	8q24.3	0.65
HSF1	8q24.3	0.63
COMMD5	8q24.3	0.62
CYC1	8q24.3	0.62
MAF1	8q24.3	0.61
GPAA1	8q24.3	0.61

Table 5 Genes with expression patterns that correlate positively with the *FUK* gene

Gene symbol	Cytoband	Spearman score
TMEM208	16q22.1	0.65
COG4	16q22.1	0.65
PRMT7	16q22.1	0.61
PSMD7	16q22.3	0.60
TRADD	16q22	0.59
WWP2	16q22.1	0.59
MTHFSD	16q24.1	0.58
CHMP1A	16q24.3	0.58
TCF25	16q24.3	0.58
ATP6V0D1	16q22.1	0.58

Table 6 Genes with expression patterns that correlate positively with the *FPGT* gene

Gene symbol	Cytoband	Spearman score
IPP	1p34-p32	0.7
ZZZ3	1p31.1	0.63
EPS15	1p32	0.62
PKN2	1p22.2	0.62
ATG4C	1p31.3	0.6
GPBP1L1	1p34.1	0.59
LEPROT	1p31.3	0.59
PIGK	1p31.1	0.59
LRRC40	1p31.1	0.58
FAM73A	1p31.1	0.58

Table 7 Genes with expression patterns that correlate positively with the *SLC35C1* gene

Gene symbol	Cytoband	Spearman score
LRG1	19p13.3	0.56
DKFZP779M0652	11p11.2	0.55
NAMPT	7q22.3	0.55
CRP	1q23.2	0.52
FNDC4	2p23.3	0.51
GLRX	5q14	0.51
LBP	20q11.23	0.51
TGM2	20q12	0.49
MGAT4B	5q35	0.48
CREB3L3	19p13.3	0.48

Table 8 Genes with expression patterns that correlate positively with the *FUT8* gene

Gene symbol	Cytoband	Spearman score
IQGAP1	15q26.1	0.71
HACD4	9p21.3	0.71
APOBEC3C	22q13.1	0.69
ELF4	Xq26	0.68
HAPLN3	15q26.1	0.68
TRERF1	6p21.1-p12.1	0.68
WIPF1	2q31.1	0.67
RAB31	18p11.3	0.66
FMNL3	12q13.12	0.66
SYT11	1q21.2	0.66

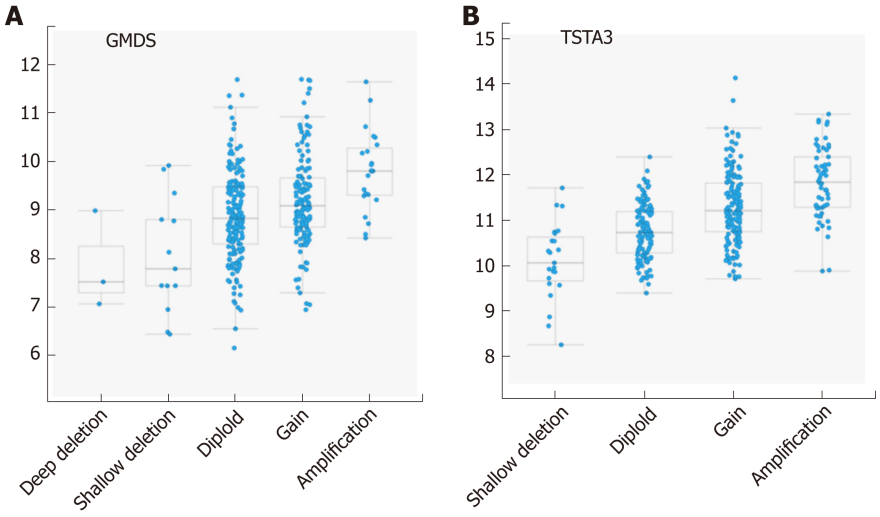


Figure 3 Correlation plots illustrating the relationship between mRNA expression levels for GMDS and TSTA3 on the Y axes (log base 2) and putative copy number changes (GISTIC2 calls, see materials and methods[26]) on the X axes. Possible GISTIC2 scores are -2 = Deep (homozygous) deletion; -1 = Shallow (hemizygous) deletion; 0 = neutral/diploid; 1 = gain (low copy); 2 = high level amplification.

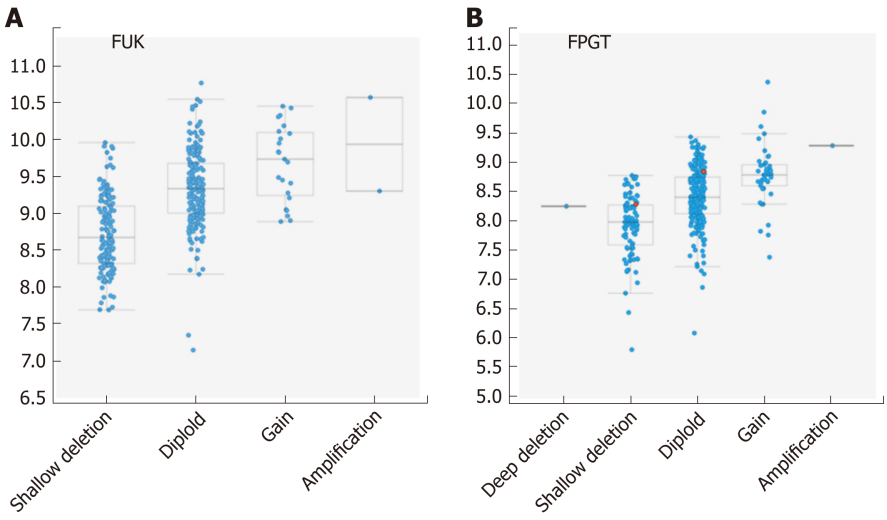


Figure 4 Correlation plots illustrating the relationship between mRNA expression levels for FUK and FPGT. Axes and scoring scale are as for Figure 3.

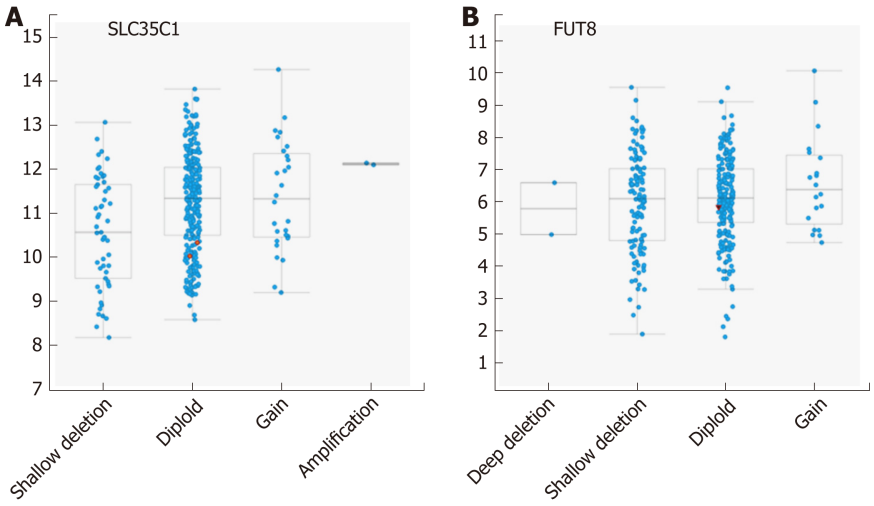


Figure 5 Correlation plots illustrating the relationship between mRNA expression levels for FUT8 and SLC35C1. Axes and scoring scale are as for Figure 3.

ARTICLE HIGHLIGHTS

Research background

One type of protein N-linked glycosylation is the addition of fucose to the innermost core N-acetylglucosamine. Increased frequency of this modification has been associated with a number of cancers, including hepatocellular carcinoma (HCC). Systematically surveying the literature as well as performing a bioinformatics survey provided insight into what has been known about the regulation of the six genes that potentially can influence levels of core fucose.

Research motivation

Knowledge of the mechanisms whereby core fucose addition is regulated may provide information to improve the utility of this protein modification as a biomarker for detection of cancer.

Research objectives

The main objective was to survey the literature for studies that include any of the six core fucose related genes in the context of HCC. The second objective was to identify genomic alterations and gene expression changes for the same six genes in HCC.

Research methods

We searched the PubMed literature database. We performed molecular analyses on the Cancer Genome Atlas Project LIHC HCC dataset, using the tools provided by the cBioportal website (http://www.cbioportal.org/).

Research results

A set of 27 non-redundant citations was generated by searching for "hepatocellular carcinoma" and each of the gene symbols TSTA3, GMDS, SLC35C1 and FUT8 or their gene products. These gene products are involved in synthesis of guanosine diphosphate (GDP)-fucose, its transport into the Golgi or its attachment to N-linked glycans. No citations mentioned genes FPGT or FUK, or their gene products, along with HCC. Analyses of the 373 sample LIHC dataset revealed limited numbers of mutations affecting protein coding regions in all six genes. However, the genes TSTA3 and GMDS that encode the enzymes of the GDP-fucose de novo synthesis pathway appeared to have undergone copy number increase in 16% and 6%, respectively, of the tumor samples. Copy number increase was observed for other genes in the vicinity of those two, which lie at 8q24 and 6p25. The other four genes tended to have increased mRNA levels in a subset of samples, which did not appear to be a consequence of copy number increase.

Research conclusions

The genes that underlie core fucose generation are not well studied in the context of HCC. Multiple molecular mechanisms appear to account for the increase in core fucosylated glycoproteins observed in some patients with HCC, with chromosomal amplification being most common.

Research perspectives

Future studies will be needed to assess whether the results observed with the LIHC dataset will be generalizable to other patient populations.

REFERENCES

1 **Debruyne EN**, Vanderschaeghe D, Van Vlierberghe H, Vanhecke A, Callewaert N, Delanghe JR. Diagnostic value of the hemopexin N-glycan profile in hepatocellular carcinoma patients. *Clin Chem* 2010; **56**: 823-831 [PMID: 20348404 DOI: 10.1373/clinchem.2009.139295]

2 **Johnson PJ**, Poon TC, Hjelm NM, Ho CS, Blake C, Ho SK. Structures of disease-specific serum alpha-fetoprotein isoforms. *Br J Cancer* 2000; **83**: 1330-1337 [PMID: 11044358 DOI: 10.1054/bjoc.2000.1441]

3 **Mehta A**, Herrera H, Block T. Glycosylation and liver cancer. *Adv Cancer Res* 2015; **126**: 257-279 [PMID: 25727150 DOI: 10.1016/bs.acr.2014.11.005]

4 **Miyahara K**, Nouso K, Dohi C, Morimoto Y, Kinugasa H, Wada N, Takeuchi Y, Kuwaki K, Onishi H, Ikeda F, Miyake Y, Nakamura S, Shiraha H, Takaki A, Amano M, Nishimura SI, Yamamoto K. Alteration of N-glycan profiles in patients with chronic hepatitis and hepatocellular carcinoma. *Hepatol Res* 2015; **45**: 986-993 [PMID: 25495282 DOI: 10.1111/hepr.12441]

5 **Miyoshi E**, Noda K, Yamaguchi Y, Inoue S, Ikeda Y, Wang W, Ko JH, Uozumi N, Li W, Taniguchi N. The alpha1-6-fucosyltransferase gene and its biological significance. *Biochim Biophys Acta* 1999; **1473**: 9-20 [PMID: 10580126 DOI: 10.1016/S0304-4165(99)00166-X]

6 **Naitoh A**, Aoyagi Y, Asakura H. Highly enhanced fucosylation of serum glycoproteins in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 1999; **14**: 436-445 [PMID: 10355508 DOI: 10.1046/j.1440-1746.1999.01882.x]

7 **Zhu J**, Wu J, Yin H, Marrero J, Lubman DM. Mass Spectrometric N-Glycan Analysis of Haptoglobin from Patient Serum Samples Using a 96-Well Plate Format. *J Proteome Res* 2015; **14**: 4932-4939 [PMID: 26448449 DOI: 10.1021/acs.jproteome.5b00662]

8 **Aoyagi Y**, Isokawa O, Suda T, Watanabe M, Suzuki Y, Asakura H. The fucosylation index of alpha-fetoprotein as a possible prognostic indicator for patients with hepatocellular carcinoma. *Cancer* 1998; **83**: 2076-2082 [PMID: 9827711 DOI: 10.1002/(SICI)1097-0142(19981115)83:10<2076::AID-CNCR5>3.3.CO;2-C]

9 **Comunale MA**, Lowman M, Long RE, Krakover J, Philip R, Seeholzer S, Evans AA, Hann HW, Block TM, Mehta AS. Proteomic analysis of serum associated fucosylated glycoproteins in the development of

primary hepatocellular carcinoma. *J Proteome Res* 2006; **5**: 308-315 [PMID: 16457596 DOI: 10.1021/pr050328x]

10 **Comunale MA**, Rodemich-Betesh L, Hafner J, Wang M, Norton P, Di Bisceglie AM, Block T, Mehta A. Linkage specific fucosylation of alpha-1-antitrypsin in liver cirrhosis and cancer patients: Implications for a biomarker of hepatocellular carcinoma. *PLoS One* 2010; **5**: e12419 [PMID: 20811639 DOI: 10.1371/journal.pone.0012419]

11 **Nie H**, Liu X, Zhang Y, Li T, Zhan C, Huo W, He A, Yao Y, Jin Y, Qu Y, Sun XL, Li Y. Specific N-glycans of Hepatocellular Carcinoma Cell Surface and the Abnormal Increase of Core-α-1, 6-fucosylated Triantennary Glycan via N-acetylglucosaminyltransferases-IVa Regulation. *Sci Rep* 2015; **5**: 16007 [PMID: 26537865 DOI: 10.1038/srep16007]

12 **Yin H**, Lin Z, Nie S, Wu J, Tan Z, Zhu J, Dai J, Feng Z, Marrero J, Lubman DM. Mass-selected site-specific core-fucosylation of ceruloplasmin in alcohol-related hepatocellular carcinoma. *J Proteome Res* 2014; **13**: 2887-2896 [PMID: 24799124 DOI: 10.1021/pr500043k]

13 **Becker DJ**, Lowe JB. Fucose: Biosynthesis and biological function in mammals. *Glycobiology* 2003; **13**: 41R-53R [PMID: 12651883 DOI: 10.1093/glycob/cwg054]

14 **Schneider M**, Al-Shareffi E, Haltiwanger RS. Biological functions of fucose in mammals. *Glycobiology* 2017; **27**: 601-618 [PMID: 28430973 DOI: 10.1093/glycob/cwx034]

15 **Cheng L**, Gao S, Song X, Dong W, Zhou H, Zhao L, Jia L. Comprehensive N-glycan profiles of hepatocellular carcinoma reveal association of fucosylation with tumor progression and regulation of FUT8 by microRNAs. *Oncotarget* 2016; **7**: 61199-61214 [PMID: 27533464 DOI: 10.18632/oncotarget.11284]

16 **Noda K**, Miyoshi E, Uozumi N, Gao CX, Suzuki K, Hayashi N, Hori M, Taniguchi N. High expression of alpha-1-6 fucosyltransferase during rat hepatocarcinogenesis. *Int J Cancer* 1998; **75**: 444-450 [PMID: 9455807 DOI: 10.1002/(SICI)1097-0215(19980130)75:3<444::AID-IJC19>3.3.CO;2-8]

17 **Wang W**, Li W, Ikeda Y, Miyagawa JI, Taniguchi M, Miyoshi E, Sheng Y, Ekuni A, Ko JH, Yamamoto Y, Sugimoto T, Yamashita S, Matsuzawa Y, Grabowski GA, Honke K, Taniguchi N. Ectopic expression of alpha1,6 fucosyltransferase in mice causes steatosis in the liver and kidney accompanied by a modification of lysosomal acid lipase. *Glycobiology* 2001; **11**: 165-174 [PMID: 11287403 DOI: 10.1093/glycob/11.2.165]

18 **Wang Y**, Fukuda T, Isaji T, Lu J, Im S, Hang Q, Gu W, Hou S, Ohtsubo K, Gu J. Loss of α1,6-fucosyltransferase inhibits chemical-induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways. *FASEB J* 2015; **29**: 3217-3227 [PMID: 25873065 DOI: 10.1096/fj.15-270710]

19 **Moriwaki K**, Noda K, Nakagawa T, Asahi M, Yoshihara H, Taniguchi N, Hayashi N, Miyoshi E. A high expression of GDP-fucose transporter in hepatocellular carcinoma is a key factor for increases in fucosylation. *Glycobiology* 2007; **17**: 1311-1320 [PMID: 17884843 DOI: 10.1093/glycob/cwm094]

20 **Noda K**, Miyoshi E, Gu J, Gao CX, Nakahara S, Kitada T, Honke K, Suzuki K, Yoshihara H, Yoshikawa K, Kawano K, Tonetti M, Kasahara A, Hori M, Hayashi N, Taniguchi N. Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. *Cancer Res* 2003; **63**: 6282-6289 [PMID: 14559815 DOI: 10.1097/00002820-200310000-00012]

21 **Tonetti M**, Sturla L, Bisso A, Benatti U, De Flora A. Synthesis of GDP-L-fucose by the human FX protein. *J Biol Chem* 1996; **271**: 27274-27279 [PMID: 8910301 DOI: 10.1074/jbc.271.44.27274]

22 **Cancer Genome Atlas Research Network**. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**: 1061-1068 [PMID: 18772890 DOI: 10.1038/nature07385]

23 **Cancer Genome Atlas Research Network**. Electronic address: wheeler@bcm.edu.; Cancer Genome Atlas Research Network. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* 2017; **169**: 1327-1341.e23 [PMID: 28622513 DOI: 10.1016/j.cell.2017.05.046]

24 **Cerami E**, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N. The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2012; **2**: 401-404 [PMID: 22588877 DOI: 10.1158/2159-8290.CD-12-0095]

25 **Gao J**, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013; **6**: pl1 [PMID: 23550210 DOI: 10.1126/scisignal.2004088]

26 **Mermel CH**, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G. GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol* 2011; **12**: R41 [PMID: 21527027 DOI: 10.1186/gb-2011-12-4-r41]

27 **Bernardi C**, Soffientini U, Piacente F, Tonetti MG. Effects of microRNAs on fucosyltransferase 8 (FUT8) expression in hepatocarcinoma cells. *PLoS One* 2013; **8**: e76540 [PMID: 24130780 DOI: 10.1371/journal.pone.0076540]

28 **Cheng L**, Luo S, Jin C, Ma H, Zhou H, Jia L. FUT family mediates the multidrug resistance of human hepatocellular carcinoma via the PI3K/Akt signaling pathway. *Cell Death Dis* 2013; **4**: e923 [PMID: 24232099 DOI: 10.1038/cddis.2013.450]

29 **Kang X**, Wang N, Pei C, Sun L, Sun R, Chen J, Liu Y. Glycan-related gene expression signatures in human metastatic hepatocellular carcinoma cells. *Exp Ther Med* 2012; **3**: 415-422 [PMID: 22969905 DOI: 10.3892/etm.2011.430]

30 **Kawamoto S**, Moriwaki K, Nakagawa T, Terao M, Shinzaki S, Yamane-Ohnuki N, Satoh M, Mehta AS, Block TM, Miyoshi E. Overexpression of α1,6-fucosyltransferase in hepatoma enhances expression of Golgi phosphoprotein 2 in a fucosylation-independent manner. *Int J Oncol* 2011; **39**: 203-208 [PMID: 21503570 DOI: 10.3892/ijo.2011.1005]

31 **Korekane H**, Hasegawa T, Matsumoto A, Kinoshita N, Miyoshi E, Taniguchi N. Development of an antibody-lectin enzyme immunoassay for fucosylated α-fetoprotein. *Biochim Biophys Acta* 2012; **1820**: 1405-1411 [PMID: 22233759 DOI: 10.1016/j.bbagen.2011.12.015]

32 **Kötzler MP**, Blank S, Bantleon FI, Spillner E, Meyer B. Donor substrate binding and enzymatic mechanism of human core α1,6-fucosyltransferase (FUT8). *Biochim Biophys Acta* 2012; **1820**: 1915-1925 [PMID: 22982178 DOI: 10.1016/j.bbagen.2012.08.018]

33 **Lee HS**, Park MH, Yang SJ, Park KC, Kim NS, Kim YS, Kim DI, Yoo HS, Choi EJ, Yeom YI. Novel candidate targets of Wnt/beta-catenin signaling in hepatoma cells. *Life Sci* 2007; **80**: 690-698 [PMID: 17157329 DOI: 10.1016/j.lfs.2006.10.024]

34 **Liu XE**, Dewaele S, Vanhooren V, Fan YD, Wang L, Van Huysse J, Zhuang H, Contreras R, Libert C, Chen CC. Alteration of N-glycome in diethylnitrosamine-induced hepatocellular carcinoma mice: A non-invasive monitoring tool for liver cancer. *Liver Int* 2010; **30**: 1221-1228 [PMID: 20524982 DOI: 10.1111/j.1478-3231.2010.02279.x]

35 **Mehta A**, Comunale MA, Rawat S, Casciano JC, Lamontagne J, Herrera H, Ramanathan A, Betesh L, Wang M, Norton P, Steel LF, Bouchard MJ. Intrinsic hepatocyte dedifferentiation is accompanied by upregulation of mesenchymal markers, protein sialylation and core alpha 1,6 linked fucosylation. *Sci Rep* 2016; **6**: 27965 [PMID: 27328854 DOI: 10.1038/srep27965]

36 **Nakagawa T**, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, Gu J, Kondo A, Taniguchi N, Miyoshi E. Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. *J Biol Chem* 2006; **281**: 29797-29806 [PMID: 16899455 DOI: 10.1074/jbc.M605697200]

37 **Okagawa Y**, Takada K, Arihara Y, Kikuchi S, Osuga T, Nakamura H, Kamihara Y, Hayasaka N, Usami M, Murase K, Miyanishi K, Kobune M, Kato J. Activated p53 with Histone Deacetylase Inhibitor Enhances L-Fucose-Mediated Drug Delivery through Induction of Fucosyltransferase 8 Expression in Hepatocellular Carcinoma Cells. *PLoS One* 2016; **11**: e0168355 [PMID: 27977808 DOI: 10.1371/journal.pone.0168355]

38 **Priyambada SA**, Misaki R, Okamoto T, Okamoto Y, Ohashi T, Ueda K, Matsuura Y, Fujiyama K. Cell surface N-glycan alteration in HepAD38 cell lines expressing Hepatitis B virus. *Virus Res* 2017; **238**: 101-109 [PMID: 28645725 DOI: 10.1016/j.virusres.2017.06.003]

39 **Wu CS**, Lee TY, Chou RH, Yen CJ, Huang WC, Wu CY, Yu YL. Development of a highly sensitive glycan microarray for quantifying AFP-L3 for early prediction of hepatitis B virus-related hepatocellular carcinoma. *PLoS One* 2014; **9**: e99959 [PMID: 24927126 DOI: 10.1371/journal.pone.0099959]

40 **Xiang T**, Yang G, Liu X, Zhou Y, Fu Z, Lu F, Gu J, Taniguchi N, Tan Z, Chen X, Xie Y, Guan F, Zhang XL. Alteration of N-glycan expression profile and glycan pattern of glycoproteins in human hepatoma cells after HCV infection. *Biochim Biophys Acta Gen Subj* 2017; **1861**: 1036-1045 [PMID: 28229927 DOI: 10.1016/j.bbagen.2017.02.014]

41 **Hutchinson WL**, Du MQ, Johnson PJ, Williams R. Fucosyltransferases: Differential plasma and tissue alterations in hepatocellular carcinoma and cirrhosis. *Hepatology* 1991; **13**: 683-688 [PMID: 1849114 DOI: 10.1002/hep.1840130412]

42 **Ji J**, Gu X, Fang M, Zhao Y, Yi C, Wang A, Gao C. Expression of alpha 1,6-fucosyltransferase 8 in hepatitis B virus-related hepatocellular carcinoma influences tumour progression. *Dig Liver Dis* 2013; **45**: 414-421 [PMID: 23352314 DOI: 10.1016/j.dld.2012.12.007]

43 **Miyoshi E**, Noda K, Ko JH, Ekuni A, Kitada T, Uozumi N, Ikeda Y, Matsuura N, Sasaki Y, Hayashi N, Hori M, Taniguchi N. Overexpression of alpha1-6 fucosyltransferase in hepatoma cells suppresses intrahepatic metastasis after splenic injection in athymic mice. *Cancer Res* 1999; **59**: 2237-2243 [PMID: 10232614]

44 **Kizuka Y**, Nakano M, Yamaguchi Y, Nakajima K, Oka R, Sato K, Ren CT, Hsu TL, Wong CH, Taniguchi N. An Alkynyl-Fucose Halts Hepatoma Cell Migration and Invasion by Inhibiting GDP-Fucose-Synthesizing Enzyme FX, TSTA3. *Cell Chem Biol* 2017; **24**: 1467-1478.e5 [PMID: 29033318 DOI: 10.1016/j.chembiol.2017.08.023]

45 **Wang L**, Huang J, Jiang M, Lin H. Tissue-specific transplantation antigen P35B (TSTA3) immune response-mediated metabolism coupling cell cycle to postreplication repair network in no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection) by biocomputation. *Immunol Res* 2012; **52**: 258-268 [PMID: 22528125 DOI: 10.1007/s12026-012-8337-z]

46 **Wang L**, Huang J, Jiang M, Lin H, Qi L, Diao H. Inhibited PTHLH downstream leukocyte adhesion-mediated protein amino acid N-linked glycosylation coupling Notch and JAK-STAT cascade to iron-sulfur cluster assembly-induced aging network in no-tumor hepatitis/cirrhotic tissues (HBV or HCV infection) by systems-theoretical analysis. *Integr Biol (Camb)* 2012; **4**: 1256-1262 [PMID: 22955522 DOI: 10.1039/c2ib20148h]

47 **Miyoshi E**, Moriwaki K, Terao N, Tan CC, Terao M, Nakagawa T, Matsumoto H, Shinzaki S, Kamada Y. Fucosylation is a promising target for cancer diagnosis and therapy. *Biomolecules* 2012; **2**: 34-45 [PMID: 24970126 DOI: 10.3390/biom2010034]

48 **Nakagawa T**, Moriwaki K, Terao N, Nakagawa T, Miyamoto Y, Kamada Y, Miyoshi E. Analysis of polarized secretion of fucosylated alpha-fetoprotein in HepG2 cells. *J Proteome Res* 2012; **11**: 2798-2806 [PMID: 22483194 DOI: 10.1021/pr201154k]

49 **Chaisaingmongkol J**, Budhu A, Dang H, Rabibhadana S, Pupacdi B, Kwon SM, Forgues M, Pomyen Y, Bhudhisawasdi V, Lertprasertsuke N, Chotirosniramit A, Pairojkul C, Auewarakul CU, Sricharunrat T, Phornphutkul K, Sangrajrang S, Cam M, He P, Hewitt SM, Ylaya K, Wu X, Andersen JB, Thorgeirsson SS, Waterfall JJ, Zhu YJ, Walling J, Stevenson HS, Edelman D, Meltzer PS, Loffredo CA, Hama N, Shibata T, Wiltrout RH, Harris CC, Mahidol C, Ruchirawat M, Wang XW; TIGER-LC Consortium. Common Molecular Subtypes Among Asian Hepatocellular Carcinoma and Cholangiocarcinoma. *Cancer Cell* 2017; **32**: 57-70.e3 [PMID: 28648284 DOI: 10.1016/j.ccell.2017.05.009]

50 **Fujimoto A**, Furuta M, Totoki Y, Tsunoda T, Kato M, Shiraishi Y, Tanaka H, Taniguchi H, Kawakami Y, Ueno M, Gotoh K, Ariizumi S, Wardell CP, Hayami S, Nakamura T, Aikata H, Arihiro K, Boroevich KA, Abe T, Nakano K, Maejima K, Sasaki-Oku A, Ohsawa A, Shibuya T, Nakamura H, Hama N, Hosoda F, Arai Y, Ohashi S, Urushidate T, Nagae G, Yamamoto S, Ueda H, Tatsuno K, Ojima H, Hiraoka N, Okusaka T, Kubo M, Marubashi S, Yamada T, Hirano S, Yamamoto M, Ohdan H, Shimada K, Ishikawa O, Yamaue H, Chayama K, Miyano S, Aburatani H, Shibata T, Nakagawa H. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet* 2016; **48**: 500-509 [PMID: 27064257 DOI: 10.1038/ng.3547]

51 **Huang W**, Skanderup AJ, Lee CG. Advances in genomic hepatocellular carcinoma research. *Gigascience* 2018; 7 [PMID: 30521023 DOI: 10.1093/gigascience/giy135]

52 **Hoshida Y**, Toffanin S, Lachenmayer A, Villanueva A, Minguez B, Llovet JM. Molecular classification and novel targets in hepatocellular carcinoma: Recent advancements. *Semin Liver Dis* 2010; **30**: 35-51 [PMID: 20175032 DOI: 10.1055/s-0030-1247131]

53 **Li X**, Xu W, Kang W, Wong SH, Wang M, Zhou Y, Fang X, Zhang X, Yang H, Wong CH, To KF, Chan SL, Chan MTV, Sung JJY, Wu WKK, Yu J. Genomic analysis of liver cancer unveils novel driver genes and distinct prognostic features. *Theranostics* 2018; **8**: 1740-1751 [PMID: 29556353 DOI: 10.7150/thno.22010]

54 **Saelee P**, Wongkham S, Bhudhisawasdi V, Sripa B, Chariyalertsak S, Petmitr S. Allelic loss on chromosome 5q34 is associated with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol*

2008; **134**: 1135-1141 [PMID: 18386060 DOI: 10.1007/s00432-008-0379-1]
55 **Yang Z**, Jiang S, Cheng Y, Li T, Hu W, Ma Z, Chen F, Yang Y. FOXC1 in cancer development and therapy: Deciphering its emerging and divergent roles. *Ther Adv Med Oncol* 2017; **9**: 797-816 [PMID: 29449899 DOI: 10.1177/1758834017742576]
56 **Frades I**, Andreasson E, Mato JM, Alexandersson E, Matthiesen R, Martínez-Chantar ML. Integrative genomic signatures of hepatocellular carcinoma derived from nonalcoholic Fatty liver disease. *PLoS One* 2015; **10**: e0124544 [PMID: 25993042 DOI: 10.1371/journal.pone.0124544]
57 **Kanai Y**, Ushijima S, Tsuda H, Sakamoto M, Hirohashi S. Aberrant DNA methylation precedes loss of heterozygosity on chromosome 16 in chronic hepatitis and liver cirrhosis. *Cancer Lett* 2000; **148**: 73-80 [PMID: 10680595 DOI: 10.1016/S0304-3835(99)00316-X]

World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 June 21; 25(23): 2961-2972

DOI: 10.3748/wjg.v25.i23.2961

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Comparison of renal safety of tenofovir and entecavir in patients with chronic hepatitis B: Systematic review with meta-analysis

Hyo-Young Lee, Hyunwoo Oh, Chan-Hyuk Park, Yee-Hui Yeo, Mindie H Nguyen, Dae-Won Jun

ORCID number: Hyo Young Lee (0000-0001-7993-3572); Hyunwoo Oh (0000-0003-4224-8808); Dae Won Jun (0000-0002-2875-6139); Chan Hyuk Park (0000-0003-3824-3481); Yee Hui Yeo (0000-0002-2703-5954); Mindie Hoai Nguyen (0000-0002-6275-4989).

Author contributions: Lee HY contributed to study concept, data search, data extraction and analysis, and drafting of the manuscript; Oh H contributed to study concept, data search, data extraction and analysis, and drafting of the manuscript; Park CH contributed to critical revision of the manuscript; Yeo YH contributed to critical revision of the manuscript; Nguyen MH contributed to supervision, revision of the manuscript; Jun DW contributed to study concept, supervision, revision of the manuscript, guarantor of the article.

Conflict-of-interest statement: The authors deny any conflict of interest.

PRISMA 2009 Checklist statement: The manuscript has been checked according to PRISMA 2009 Checklist.

Hyo-Young Lee, Hyunwoo Oh, Dae-Won Jun, Department of Internal Medicine, Hanyang University College of Medicine, Seoul 04763, South Korea

Chan-Hyuk Park, Department of Internal Medicine, Hanyang University Guri Hospital, Hanyang University College of Medicine, Guri 11923, South Korea

Yee-Hui Yeo, Mindie H Nguyen, Division of Gastroenterology and Hepatology, Stanford University Medical Center, Stanford University, Stanford 94305, CA, United States

Corresponding author: Dae-Won Jun, MD, PhD, Professor, Department of Internal Medicine, Hanyang University College of Medicine, 17 Haengdang-dong Sungdong-gu, Seoul 04763, South Korea. noshin@hanyang.ac.kr
Telephone: +82-2-22908338
Fax: +82-2-9720068

Abstract

BACKGROUND

Recently, the American Association for the Study of Liver Disease suggested no preference between tenofovir (TDF) and entecavir (ETV) regarding potential long-term risks of renal complications. Over the years, renal safety has become a critical concern in nucleos(t)ide analog-treated patients due to the long-term use of these drugs. However, existing studies do not show significant differences in renal dysfunction between these two drugs. Further, there is a paucity of studies comparing the long-term renal effects of TDF and ETV.

AIM

To investigate the effects of TDF and ETV on renal function, we performed systematic review and meta-analysis.

METHODS

Two investigators independently searched the Cochrane Library, MEDLINE, and Embase databases for randomized controlled trials and nonrandomized studies (NRSs) using the keywords "CHB", "Tenofovir", and "Entecavir", and additional references were obtained from the bibliographies of relevant articles published through December 2017. The quality of each study was assessed using the Newcastle-Ottawa scale and the Grading of Recommendations Assessment, Development and Evaluation criteria. The primary outcome was the change in serum creatinine level in the TDF and ETV groups at baseline, 6 mo, 12 mo and 24 mo.

RESULTS

Manuscript source: Unsolicited manuscript

Received: March 22, 2019
Peer-review started: March 22, 2019
First decision: April 4, 2019
Revised: April 27, 2019
Accepted: May 8, 2019
Article in press: May 8, 2019
Published online: June 21, 2019

P-Reviewer: El-Razek AA, Hann HW, Yoshioka K
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

Nine NRSs comprising 2263 participants met the inclusion criteria. Changes in creatinine levels were higher in the TDF group than in the ETV group at 6 mo [mean difference (MD) = 0.03 mg/dL; 95%CI: 0.02-0.04; I^2 = 0%], 12 mo (MD = 0.05 mg/dL; 95%CI: 0.02-0.08; I^2 = 78%), and 24 mo (MD = 0.07 mg/dL; 95%CI: 0.01-0.13; I^2 = 93%). The change in estimated glomerular filtration rate (eGFR) was significantly higher in the TDF group than in the ETV group at 6 mo [standardized mean difference (SMD), -0.22; 95%CI: -0.36--0.08; I^2 = 0%], 12 mo (SMD = -0.24; 95%CI: -0.43--0.05; I^2 = 50%), and 24 mo (-0.35; 95%CI: -0.61- -0.09; I^2 = 67%).

CONCLUSION

TDF statistically significantly increased serum creatinine levels and decreased the eGFR in 6-24 mo compared to ETV, with moderate to low quality of evidence. However, the differences are negligible.

Key words: Hepatitis B; Chronic; Tenofovir; Entecavir; Safety; Review; Systematic; Meta-analysis

Core tip: Recently, the American Association for the Study of Liver Disease suggested no preference between tenofovir (TDF) and entecavir (ETV) regarding potential long-term risks of renal complications. Over the years, renal safety has become a critical concern in nucleos(t)ide analog-treated patients due to the long-term use of these drugs. However, the existing studies do not show significant differences between the two drugs in renal dysfunction. We believe that our study could resolve the existing debate. This is the first meta-analysis comparing the influence of the two drugs on renal function using continuous variables. TDF statistically significantly decreases renal function compared to ETV, but the difference is inappreciable.

Citation: Lee HY, Oh H, Park CH, Yeo YH, Nguyen MH, Jun DW. Comparison of renal safety of tenofovir and entecavir in patients with chronic hepatitis B: Systematic review with meta-analysis. *World J Gastroenterol* 2019; 25(23): 2961-2972
URL: https://www.wjgnet.com/1007-9327/full/v25/i23/2961.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i23.2961

INTRODUCTION

An estimated 257 million people worldwide are infected with the hepatitis B virus (HBV)[1]. Recently, research addressing chronic kidney disease among chronic hepatitis B patients has emerged[2]. Interest in drugs that can affect renal function has also increased. Tenofovir (TDF) and entecavir (ETV) are two drugs preferred as first-line treatments for chronic hepatitis B (CHB). In clinical trials, both have been proven to be safe and well-tolerated with short term follow-up; however, as the treatment period is indefinite, adverse events associated with these drugs over the long term remain worrisome[3,4]. Over the years, published studies have provided increasing evidence that TDF can negatively influence renal function and bone health[5-7]. Further confusing the issue, two recently announced guidelines make conflicting suggestions. The European Association for the Study of the Liver (EASL) recommended considering switching from TDF to ETV in CHB patients with underlying renal disease, especially when exposed to LAM. EASL also suggested selecting ETV (or tenofovir alafenamide fumarate) over TDF for CHB patients with estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2, patients with albuminuria, and patients on hemodialysis. However, the American Association for the Study of Liver Disease (AASLD) suggested no preference between TDF and ETV use with regard to renal safety issues[3,4].

Most physicians acknowledge renal concerns associated with TDF use, although precise data on the degree of renal impairment are still limited. Results of previous studies have varied, mainly limited by small sample sizes and inadequate follow-up periods. Previous meta-analyses on antiviral therapy for CHB primarily focused on therapeutic efficacy rather than renal safety issues[8-10]. Hence, data regarding

creatinine level and eGFR were not reported comprehensively. Moreover, the formulas for eGFR calculation were inconsistent, as some studies used the Modification of Diet in Renal Disease (MDRD) formula, while others used the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation[8,9]. In addition, previous studies demonstrated the incidence of acute kidney injury (AKI) as dichotomous data but not the precise changing values of renal function during the treatment period.

In this study, we aimed to conduct a systematic review and meta-analysis to provide a clear comparison of the renal safety of TDF and ETV in patients with CHB using continuous variables. To enhance the clinical importance, we performed subgroup analyses and sensitivity analysis to determine changes in renal function by study and patient characteristics.

MATERIALS AND METHODS

This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Review and Meta-analyses (PRISMA) statement[11] and Metaanalysis of Observational Studies in Epidemiology (MOOSE) statements[12].

Data and literature sources

Two authors (Lee HY and Oh H) independently searched MEDLINE, Embase, and the Cochrane Central Register of Controlled Trials (CENTRAL) from inception to December 31, 2017, without language restriction. Additionally, we examined conference abstracts from the AASLD, the EASL, the Asian Pacific Association for the Study of the Liver (APASL), Digestive Disease Week (DDW), and The Liver Weeks (Korean Association for the Study of the Liver) published between 2013 and 2017 (Supplemental Table 1).

The following keywords, MeSH, and free text were searched through MEDLINE: Tenofovir, Entecavir, chronic hepatitis B, and multiple synonyms (Supplemental Table 2). After the initial electronic search, further relevant articles and bibliographies were manually identified using reference lists from included studies. The identified articles were assessed individually for inclusion (Supplemental Table 3).

Inclusion and exclusion criteria

Two authors (Lee HY and Oh H) independently identified articles eligible for inclusion based on a two-level screening using the Population Intervention Comparison Outcome (PICO) framework[13]. At the first level, titles and abstracts were screened, while at the second level, search queries of the full text of articles were made. The observed agreement between reviewers for eligibility of articles on initial screening was 98.2%, corresponding to substantial agreement (k = 0.79), and that in the second screening was 94.6%, corresponding to almost perfect agreement (k = 0.89). Disagreements between reviewers were resolved by consensus with a third author (Jun DW).

The inclusion criteria were (1) Human subject study design, including randomized control trials (RCTs) and non-RCTs with more than two arms, (2) CHB infection, (3) Intervention therapies of either ETV or TDF monotherapy, (4) Over 6 months of treatment duration, (5) Over 18 years of age, and (6) Documented data of repeated measures of serum creatinine level or eGFR every six months.

Exclusion criteria were (1) Coinfection with other hepatitis viruses (A, C, D, or E), human immunodeficiency virus, cytomegalovirus, or Epstein-Barr virus; (2) Other liver diseases such as alcoholic liver disease, autoimmune hepatitis, drug-induced liver injury, or Wilson's disease; (3) Acute hepatitis and acute exacerbation; (4) Combination therapy or sequential therapy; (5) Unreported renal parameter data (serum creatinine or eGFR); (6) Exclusion of either TDF or ETV; (7) Pregnancy and/or breastfeeding; (8) Complication of decompensated cirrhosis (variceal bleeding, refractory ascites, hepatorenal syndrome, spontaneous bacterial peritonitis, hepatoencephalopathy); and (9) Organ transplantation.

Data extraction

Two authors (Lee HY and Oh H) independently extracted data from each study with a predefined data extraction form using the Cochrane Methods to minimize random and bias errors. Any disagreement or unresolved concern was independently reviewed by a third author (Jun DW). The following variables were extracted from the selected studies: (1) To evaluate the renal side effect, we compared changes in serum creatinine level and eGFR at baseline, 6 mo, 12 mo and 24 mo. The patient's eGFR was calculated using the MDRD formula and CKD-EPI equation (Supplemental Table 4).

Table 1 Main characteristics of included studies

Author, year country	Design	Duration (mo)	Cirrhosis (%)	Patients (*n*)	Age (mean)	Sex (M/F)	HBV_DNA	End point
				TDF	TDF	TDF		
				ETV	ETV	ETV	(log10 IU/Ml)	
Ha *et al*[14] 2015 United States	Matched case-cohort	24(18-66)	9.20%	103	43.5	65/38	5.3 ± 1.5	RMSRC Decrease in eGFR 20%
				103	43.8	65/38	6.15 ± 1.9	
Lee *et al*[15] 2015 South Korea	Prospective	≥ 6	NA	258	66.4	173/85	6.40 ± 1.31	NA
				308	50.5	190/118	6.72 ± 1.22	
Yu *et al*[16] 2015 South Korea	Retrospective	8.45/18.7	50%	49	48.8	22/27	6.98 ± 1.55	Creatinine ≥ 0.3 mg/dL or 1.5 times above baseline
				58	51.7	33/25	7.05 ± 1.33	
Park *et al*[20] 2017 South Korea	Retrospective	24	100%	73	56.4	45/28	5.4 ± 1.3	Serum creatinine increase > 0.2 mg/dLeGFR < 60 mL/min (CKD-EPI)Decrease in eGFR > 20% (CKD-EPI)
				162	55.6	110/52	5.6 ± 1.6	
Koklu *et al*[24] 2015 Turkey	Prospective	24	34%	273	47.7	183/90	6.54 ± 1.74	Shift from ≥ 90 to 60-89 mL/min per 1.73 m^2> 25% increased creatinine
				282	49.9	197/85	6.69 ± 1.79	
Idilman *et al*[25] 2015 Turkey	Retro-prospective	36 (6-78)	39%	172	47 (15-79)	NA	5.97 ± 1.72	Serum creatinine > 0.5 mg/dL from baseline, eGFR < 50 mL/min
				183		NA		
Koksal 2016 *et al*[23] Turkey	Prospective	≥ 24	NA	44	36 (29-43.7)	19/25	6.8 ± 1.0	
				32	40 (27.2-46.5)	17/15	7.0 ± 1.2	
Lopez *et al*[22] 2016 Spain	Retrospective	12	33.90%	32	50.2	25/7	1127.4 (19-2463,121)	eGFR < 60 mL/min per 1.73 m^2 eGFR reduction > 25%Increase in creatinine > 1.4 mg/dl
				32	49.2	23/9	29311.4 (376.2-4660,135)	
Tsai *et al*[21] 2016 Taiwan	Retro-prospective	≥ 24	52%	37	56.6	32/5	6.3 ± 1.3	Change of eGFR by 25%
				62	55.2	46/16	6.4 ± 1.2	

TDF: Tenofovir; ETV: Entecavir; HBV: Hepatitis B virus; CHB: Chronic hepatitis B; M: Male; F: Female; NA: Not available; eGFR: Estimated glomerular filtration rate; HTN: Hypertension; DM: Diabetes mellitus; HCC: Hepatocellular carcinoma; MDRD: Modification of diet in renal disease; CKD-EPI: Chronic kidney disease epidemiology collaboration; CTx: Chemotherapy; OT: Organ transplantation; RMSRC: Reclassified to a more severe renal classification.

All outcomes were assessed for changes due to intervention between treated and control groups. The results are expressed as the means and standard deviations. (2) When the study presented data on renal function using a graph rather than measured numerical data, we extracted comparable data from the graph. (3) When the data of interest were not available in the published reports, we contacted investigators of original studies via e-mail to request unpublished data, and 3 investigators responded to our request[14-16]. And (4) If necessary, we modified the data (combining two data or converting standard error to standard deviation) to enable comparison according to the equation presented in the Cochrane Handbook (Supplemental Table 5)[17].

Assessment of methodological quality

Two authors (Lee HY and Oh H) independently evaluated the quality of the included studies using the Newcastle-Ottawa scale (NOS) for nonrandomized studies (Supplemental Table 6)[18] and the Grading of Recommendations Assessment, Development and Evaluation (GRADE) criteria to appraise quality of evidence (GRADEpro, Version

20. McMaster University, 2014) (Supplemental Table 7)[19]. Any disagreements between reviewers were resolved through discussion or review by the third author (Jun DW).

Statistical analysis

The primary outcome was the change in serum creatinine levels in the TDF and ETV groups at baseline, 6 mo, 12 mo and 24 mo. We derived the mean differences in creatinine levels between the aforementioned time points and the baseline. Subsequently, we estimated and pooled the differences in the mean between the TDF and ETV groups using a random effects model. A significant difference was defined as having a *P*-value of Z-score smaller than 0.05. To assess for heterogeneity, we estimated the proportion of inconsistencies due to true differences between studies (rather than differences due to random error or chance) using the I^2 statistic, with values of 25%, 50%, and 75% considered low, moderate, and high, respectively. Publication bias was assessed by funnel plots and Egger's test.

The secondary outcome was the change in serum eGFR. As eGFR was estimated by different formulas (*e.g.*, MDRD or CKD-EPI), we standardized the mean difference of eGFR in each included article before pooling them using a random effects model.

Subgroup meta-analyses stratified by proportion of cirrhosis, history of treatment, region, mean age, and other factors were performed subsequently. Meta-regression was performed to identify the source of heterogeneity and to investigate the nature of the studies required to estimate the therapeutic effect. Meta-analyses were performed using the Review Manager (RevMan, version 5.3; The Cochrane Collaboration, Oxford, United Kingdom). Publication bias and meta-regression were analyzed using Comprehensive Meta-Analysis (CMA) statistical software (Version 3, BioStat Solutions, Inc.).

RESULTS

Identification of studies

Figure 1 shows the details of the literature search and study selection. The initial search strategy identified 5413 articles (Supplemental Table 3). Of these, 5327 publications were excluded, as they did not fulfill the selection criteria by title and abstract screening. We performed full manuscript reviews of the remaining 86 articles. Nine reports (three prospective cohort studies and six retrospective cohort studies) were deemed eligible and included in the meta-analysis[14-16,20-25].

Study characteristics and patient populations

Table 1 describes the characteristics of the 9 included studies. A total of 2263 participants who received either TDF (n = 1041) or ETV (n = 1222) were included. Both drugs were administered for 6 mo to 2 years. Three studies were conducted in South Korea; one each in United States, Taiwan, and Spain; and three in Turkey. Serum creatinine data were provided in 9 studies and eGFR data in 6 studies. Except for one study[22], all of the studies recruited treatment-naive patients.

Quality of the included studies

The quality of evidence was assessed using the NOS and GRADE guidelines. The level of evidence and grade of recommendation for each outcome are summarized in Supplemental Table 6 and 7.

Primary outcome: Change in serum creatinine

Renal function was assessed using serum creatinine levels at 6, 12, and 24 mo of treatment and its decrease from baseline. Using a random effects approach, changes in serum creatinine level compared to baseline increased more in the TDF group than in the ETV group at 6 mo (MD = 0.03 mg/dL; 95%CI: 0.02-0.04; I^2 = 0%), 12 mo (MD = 0.05 mg/dL; 95%CI: 0.02- 0.08; I^2 = 78%), and 24 mo (MD = 0.07 mg/dL; 95%CI: 0.01-0.13; I^2 = 93%) (Figure 2). The subgroup difference among different time points was not significant.

Secondary outcome: Changes of eGFR

Changes in eGFR were significantly higher in the TDF group than in the ETV group. Most studies used various eGFR formulas (Supplement Table 8). The standardized mean differences (SMDs) of serum eGFR between the TDF and ETV groups at 6, 12, and 24 mo were -0.22 (95%CI: -0.36--0.08; I^2 = 0%), -0.24 (95%CI: -0.43--0.05; I^2 = 50%), and -0.35 (95%CI: -0.61--0.09; I^2 = 67%), respectively (Figure 3). There was no significant difference in SMD among different time points.

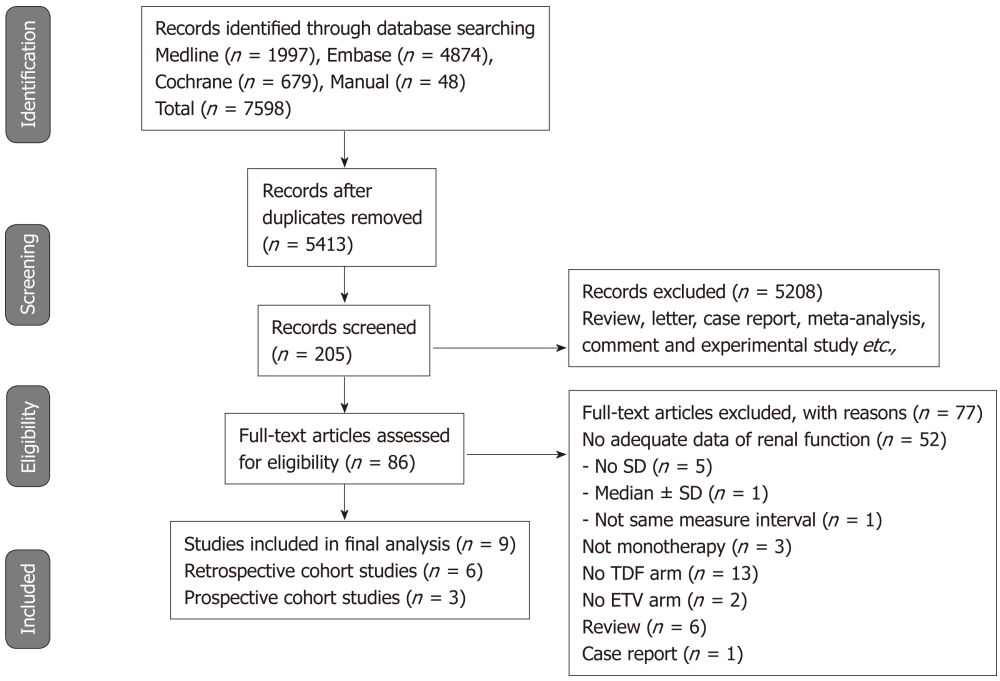


Figure 1 PRISMA diagram of the literature search. TDF: Tenofovir; ETV: Entecavir; SD: Standard deviation.

Subgroup analysis

To assess the effect size, we conducted subgroup analysis of calculated MDRD and CKD-EPI after securing raw data on creatinine (Figure 4). The MDRD and CKD-EPI equations both showed the same directivity without subgroup difference in each follow-up period (I^2 = 0%). However, the studies used for the analysis of MDRD and CKD-EPI were different, and the number of studies was insufficient.

Sensitivity analysis

We tested the heterogeneity of studies with 12-mo and 24-mo enrollment categories, as shown in Figure 2. To determine the cause of heterogeneity, we compared the inclusion criteria of the enrolled studies (Supplemental Table 8) and conducted meta-regression (Figure 5A and B). We considered the possibility that (mean age, creatinine) may have been a major source of substantial heterogeneity observed across the studies by research-level factors. In the meta-regression analysis, we did not find any statistically significant tendency dependent on available study characteristics. However, individual patient-level data could have been weak due to aggregation bias. But through meta-regression we identified two studies as potential sources of heterogeneity. Park *et al*[20] analyzed only cirrhotic patients in their study, and included 22% of decompensated cirrhotic patients, diabetics and patients who take diuretics. Tsai *et al*[21] showed greater heterogeneity than other studies. Tsai included patients with all impaired renal function (eGFR 30-90 mL/min per 1.73). When we excluded the study by Tsai *et al*[21], heterogeneity decreased from 78% to 1% in the 12-mo group and from 93% to 82% in the 24-mo group. After excluding the Tsai *et al*[21] study, the effect size remained statistically significant (Figure 5C).

Publication bias

Funnel plots constructed with observed studies showed symmetry (Figure 6). In Egger's test, publication bias revealed no significant evidence [bias = 1.967 (95%CI: -2.067-5.980), P = 0.285]. None of the studies trimmed in the Trim & Fill method with the random effect model. There was no significant publication bias detected by funnel plots.

DISCUSSION

In this systematic review and meta-analysis of prior studies, (1) The mean differences in serum creatinine levels between TDF and ETV were 0.03 mg/dL (95%CI: 0.02-0.04; I^2 = 0%), 0.05 mg/dL (95%CI: 0.02 to 0.08; I^2 = 78%), and 0.07 mg/dL (95%CI: 0.01-0.13; I^2 = 93%) at 6, 12, and 24 mo, respectively. Although the mean difference was statistically significant, a gap of 0.03-0.07 mg/dL does not reach clinical significance.

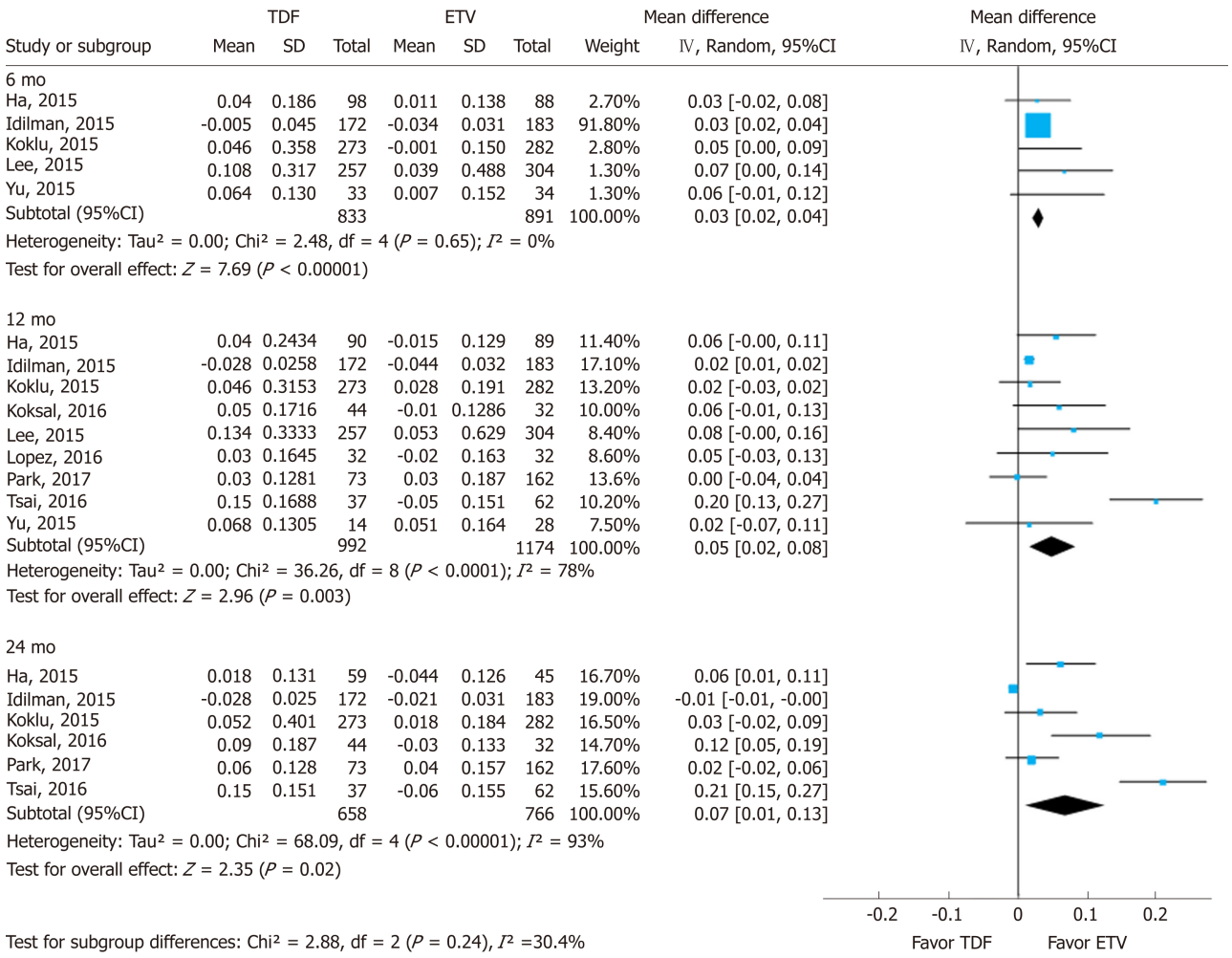


Figure 2 Forest plot for the change of serum creatinine. In each enrolled study, the change (between post-treatment and baseline) is calculated in each arm [tenofovir (TDF) *vs* entecavir (ETV)]. The difference in mean change between the two changes (δ TDF - δ ETV) is then calculated. TDF: Tenofovir; ETV: Entecavir; SD: Standard deviation; CI: Confidence interval.

Although the mean difference seemed to increase gradually over time, there was no significant difference among the estimates. (2) Similarly, there was a significant standardized mean difference in serum eGFR levels between the two drugs over the same time periods. TDF showed a greater decrease than ETV after 6, 12, and 24 mo use. And (3) No significant difference was found through subgroup analysis and sensitivity analysis by research-level factors and patient-level factors.

Our research is different from existing studies for the following reasons: (1) We used continuous variables when comparing the influence of drugs on renal function. (2) We enhanced clinical relevance by using qualified research quality assessment methods and sensitivity analysis with meta-regression by study and patient characteristics. Although there have been many studies and efforts to clarify the influence of anti-HBV agents on renal function, these have resulted in conflicting data[8-10]. To our knowledge, there were three systematic reviews for efficacy and safety comparing TDF and ETV, but two of them primarily focused on efficacy rather than renal safety issues[8,9]. Some critical manuscripts and abstracts were not included in the previous studies. Moreover, most studies used various eGFR formula and different AKI definitions. Lok *et al*[9] only listed dichotomous data that consisted of an arbitrary definition of deterioration of renal function by NAs and change in eGFR values without statistical analysis. Han *et al*[8] suggested more complex conclusions. In their work, there was a statistically significant difference between TDF and ETV in eGFR at the endpoint (RR = 1.601, 95%CI: 1.035-2.478, I^2 = 0.0%), but no significant difference in the change of eGFR from baseline (RR = 0.929, 95%CI: 0.616-1.4601, I^2 = 0.0%). However, the authors did not clearly show how many studies were included in the analysis. Because renal safety was defined as a secondary outcome, there were several missing papers, and selection bias might have occurred. Moreover, in case of the study by Gish *et al*, which was one of the studies included by Han *et al*[8], nephrotoxicity was defined by an increase in serum creatinine of ≥ 0.2 mg/dL. There

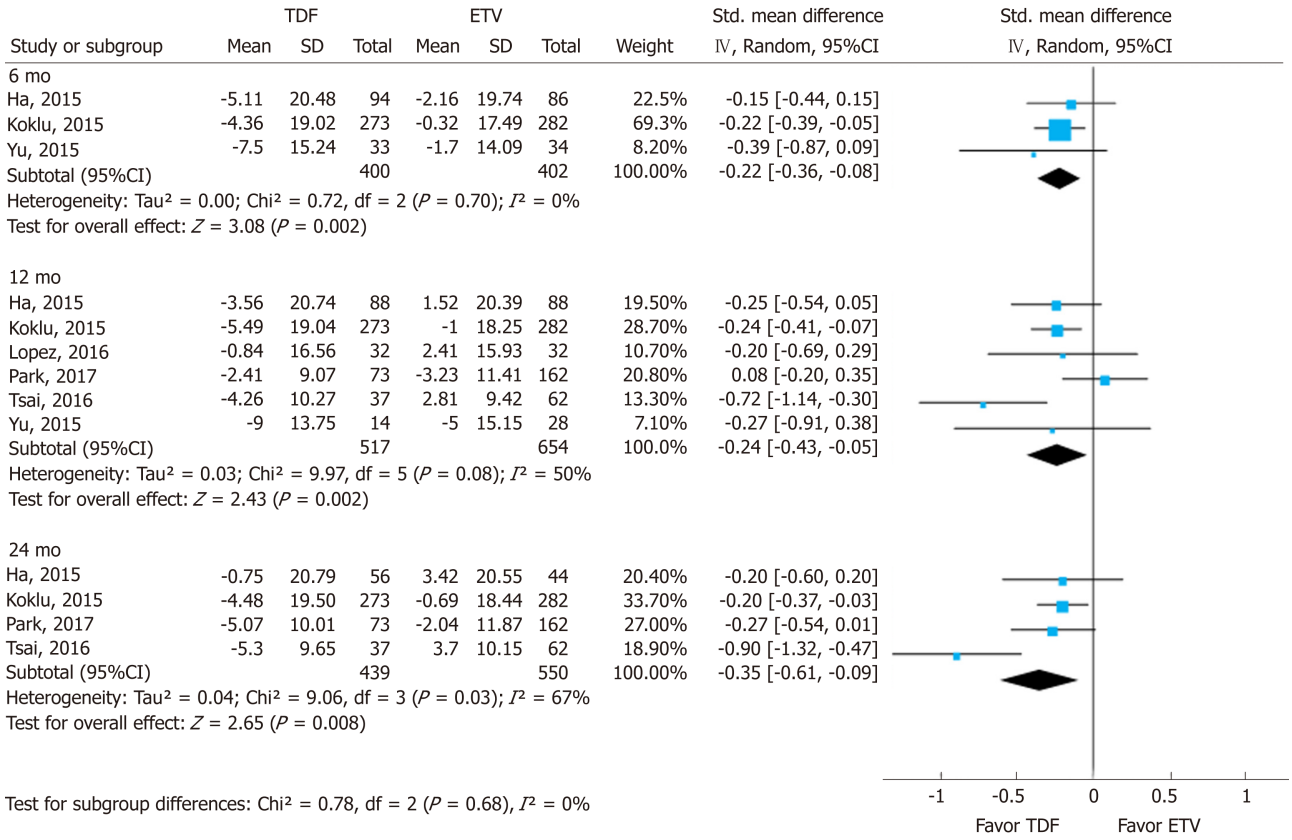


Figure 3 Forest plot for the change of estimated glomerular filtration rate. In each enrolled study, the change (between post-treatment and baseline) is calculated in each arm [tenofovir (TDF) *vs* entecavir (ETV)]. The difference in standardized mean change between the two changes (δ TDF - δ ETV) is then calculated, since each study utilized different formulas. TDF: Tenofovir; ETV: Entecavir; SD: Standard deviation; CI: Confidence interval.

was no difference between the TDF- and ETV-treated groups, but more patients in the TDF group experienced eGFR decreases than in the ETV group. In this case, changes in renal function could be underestimated. An article by Chan *et al*[10] showed effect size with a continuous variable. They compared the effect of various NAs on renal function using a network analysis. However, the authors did not include articles regarding the effects of NAs on renal function comprehensively, with only three articles making direct comparisons between TDF and ETV. Moreover, the definition of AKI was inconsistent in the three included articles.

In this study, we determined that while TDF has been linked to declines in renal function, the difference is not clinically definitive. Although there was a statistically significant decrease in the short- and medium-term, our study does not guarantee long-term stability. Moreover, some studies found no significant change in renal function in TDF users in the longer term[26-28]. In sensitivity analysis, we conjectured that the use of TDF is associated with mild renal impairment. Although there are very few studies on CKD patients, many studies have performed a subgroup analysis of CKD patients in CHB. Recently, Trinh *et al*[29] showed that in the absence of underlying disease, the use of TDF did not significantly impair renal function, but the use of TDF in patients with CKD and over 60 years of age exacerbated a decline in renal function. In the study by Wong *et al*[30], the TDF-treated group had a significantly increased risk of CKD progression (HR = 1.21) and showed a more rapid progression of CKD.

Our study has several limitations: (1) Using GRADE criteria, we found that overall confidence in estimates was low. Due to the nature of the nonrandomized designs used in these studies, there was serious inconsistency and the level of quality of most of the studies was low (Supplemental Table 7). (2) The heterogeneity of several subgroups were high. Through the sensitivity analysis, we found the weight and effect size of one specific paper was large and have tried to explain the reason for this specificity. (3) We used SMD to perform the meta-analysis, as the formulas for eGFR calculation varied across studies. Therefore, the results need to be interpreted with caution. (4) There was a fundamental clinical heterogeneity. Although we performed sensitivity analysis, Child-Turcotte-Pugh score, hepatocellular carcinoma, hypertension, and diabetes mellitus were not controlled perfectly. (5) Our findings should be interpreted cautiously, since the studies included in our meta-analysis adopted

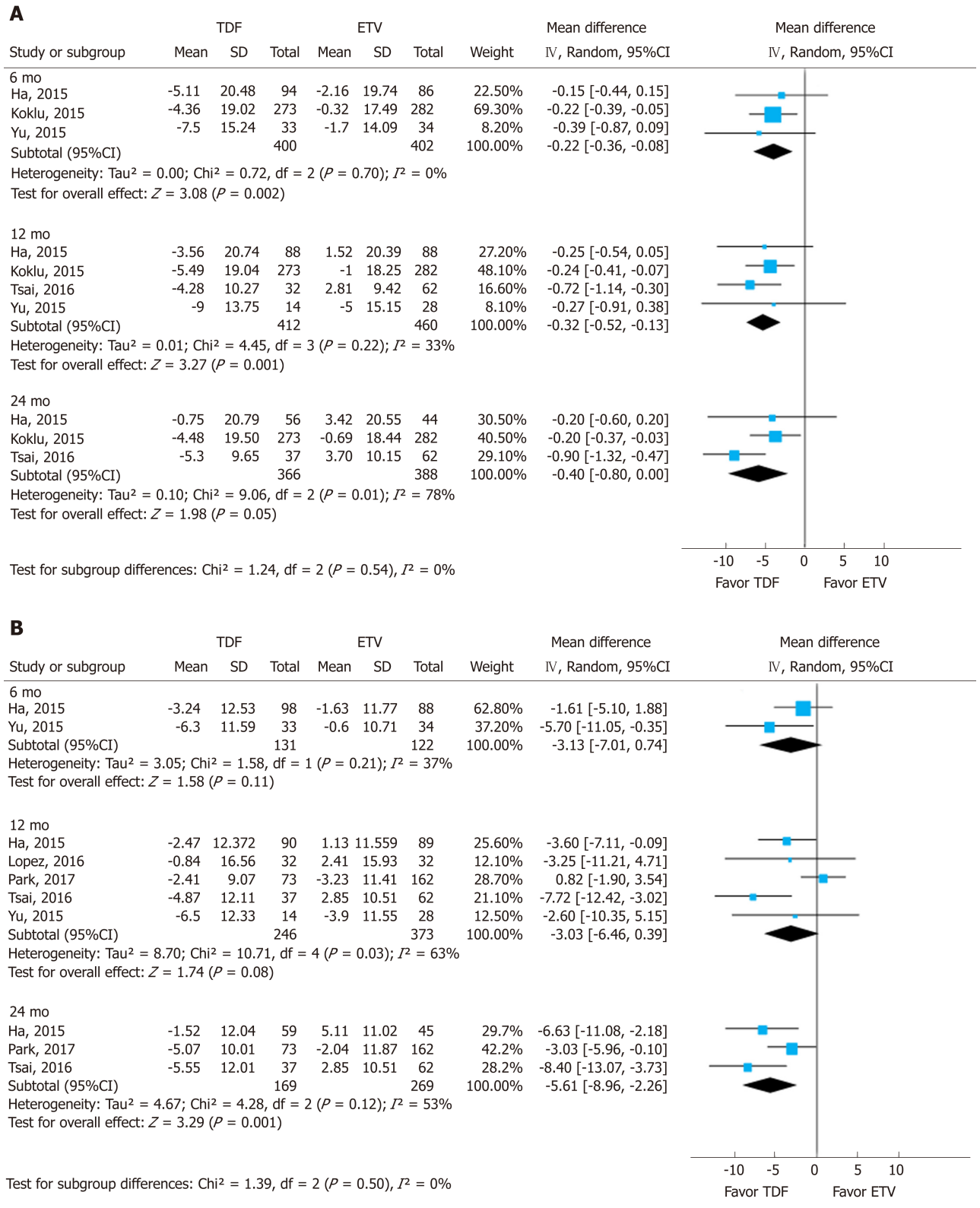


Figure 4 Subgroup analysis. Likewise, the change is calculated using a common formula in each arm to assess the effect size. A: Modification of diet in renal disease, B: Chronic kidney disease epidemiology collaboration. TDF: Tenofovir; ETV: Entecavir; SD: Standard deviation; CI: Confidence interval.

different definitions and inclusion criteria for renal impairment. And (6) Finally, the total number of studies and patients is small. Larger scale cohort studies with long-term follow-up are warranted to provide more precise data on long-term renal adverse events.

In summary, our meta-analysis of observational studies reveals that TDF statistically significantly increased serum creatinine levels and decreased the eGFR over 6-24 mo in comparison with ETV. However, the quality of the evidence was moderate to low quality and the difference was inappreciable.

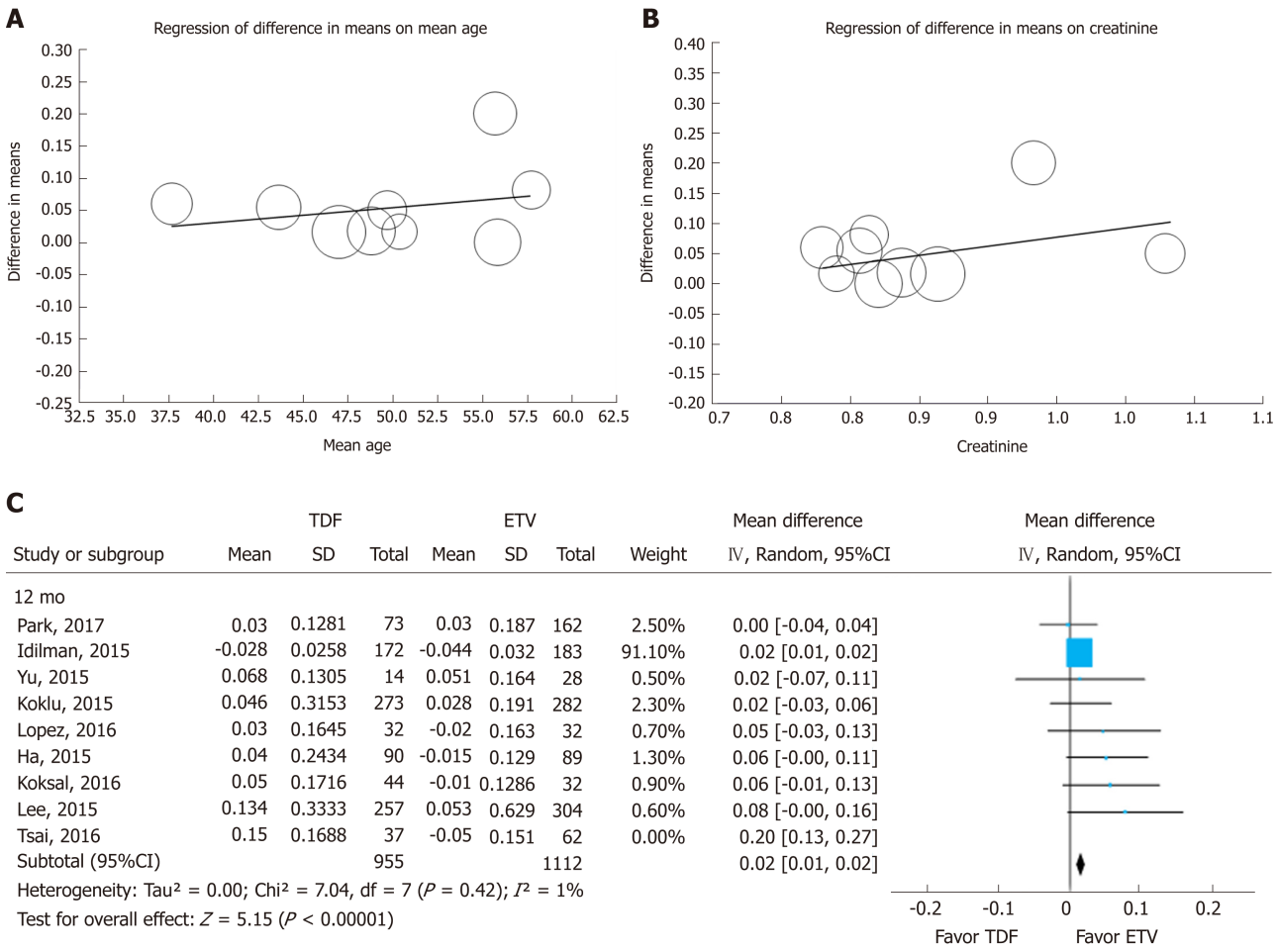


Figure 5 Sensitivity analysis. Meta-regression of difference in mean age (A) and creatinine (B). The circles on the graph represent included studies; the size of the circle indicates the weight of each study. To check the robustness of this study, we tentatively excluded one heterogeneous study[19] (C). TDF: Tenofovir; ETV: Entecavir; SD: Standard deviation; CI: Confidence interval.

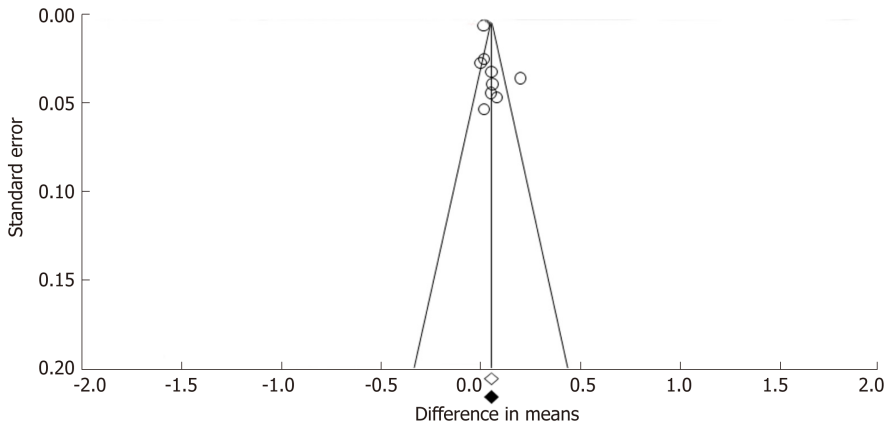


Figure 6 Funnel plot for publication bias in all included studies. The empty circles represent the observed studies and empty and black-filled diamonds represent the overall random effects means and 95% confidence intervals of the observed studies.

ARTICLE HIGHLIGHTS

Research background

Tenofovir (TDF) and entecavir (ETV) are preferred first-line treatments for chronic hepatitis B (CHB). The long-term safety issue of nucleos(t)ide analog is very important because CHB patients should take it indefinitely. In addition, a number of researchers have recently reported many CHB patients suffer from CKD through large-scale studies.

Research motivation
Over the years, several studies have been conducted to compare renal safety of the two drugs, but the results varied and sometimes conflicted with each other. Confusedly, the recommendations of the recent two guidelines are contradictory.

Research objectives
We aimed to conduct a systematic review and meta-analysis to assess renal safety of TDF and ETV in patients with CHB using continuous variables.

Research methods
Calculating the change of creatinine and estimated glomerular filtration rate (eGFR), we secured the distinction from the prior meta-analysis that using dichotomous data. To enhance the clinical importance, we performed sensitivity analysis with meta-regression.

Research results
With nine NRSs, we conducted meta-analysis. Changes in creatinine levels were higher in the TDF group than in the ETV group at 6 mo [mean difference (MD) = 0.03 mg/dL; 95%CI: 0.02-0.04; I^2 = 0%], 12 mo (MD = 0.05 mg/dL; 95%CI: 0.02 to 0.08; I^2 = 78%), and 24 mo (MD = 0.07 mg/dL; 95%CI: 0.01-0.13; I^2 = 93%). The change in eGFR was significantly higher in the TDF group than in the ETV group at 6 mo [standardized mean difference (SMD) = -0.22; 95%CI: -0.36--0.08; I^2 = 0%], 12 mo (SMD = -0.24; 95%CI: -0.43 to -0.05; I^2 = 50%), and 24 mo (-0.35; 95%CI: -0.61--0.09; I^2 = 67%).

Research conclusions
Until now, in studies comparing the effect on renal function between the two drugs, the differences varied greatly. However, our study found that the difference was negligible.

Research perspectives
The value of creatinine and eGFR in our meta-analysis was a secondary outcome in most of the included studies. And most of the studies used various eGFR formula and different AKI definitions. We need further research comparing renal function as a primary outcome and using universal definition of AKI, if possible, through large-scale RCT.

REFERENCES

1 **World Health Organization**. Global Hepatitis Report, 2017. Geneva, Switzerland: World Health Organization. Available from: https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/
2 **Nguyen MH**, Lim JK, Burak Ozbay A, Fraysse J, Liou I, Meyer N, Dusheiko G, Gordon SC. Advancing Age and Comorbidity in a US Insured Population-Based Cohort of Patients With Chronic Hepatitis B. *Hepatology* 2019; **69**: 959-973 [PMID: 30175482 DOI: 10.1002/hep.30246]
3 **Terrault NA**, Lok ASF, McMahon BJ, Chang KM, Hwang JP, Jonas MM, Brown RS, Bzowej NH, Wong JB. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology* 2018; **67**: 1560-1599 [PMID: 29405329 DOI: 10.1002/hep.29800]
4 **European Association for the Study of the Liver;**. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* 2017; **67**: 370-398 [PMID: 28427875 DOI: 10.1016/j.jhep.2017.03.021]
5 **Wang HM**, Hung CH, Lee CM, Lu SN, Wang JH, Yen YH, Kee KM, Chang KC, Tseng PL, Hu TH, Chen CH. Three-year efficacy and safety of tenofovir in nucleos(t)ide analog-naïve and nucleos(t)ide analog-experienced chronic hepatitis B patients. *J Gastroenterol Hepatol* 2016; **31**: 1307-1314 [PMID: 26758501 DOI: 10.1111/jgh.13294]
6 **Petersen J**, Heyne R, Mauss S, Schlaak J, Schiffelholz W, Eisenbach C, Hartmann H, Wiese M, Boeker K, Loehr HF, John C, Leuschner M, Trautwein C, Felten G, Trein A, Krause W, Ruppert S, Warger T, Hueppe D. Effectiveness and Safety of Tenofovir Disoproxil Fumarate in Chronic Hepatitis B: A 3-Year Prospective Field Practice Study in Germany. *Dig Dis Sci* 2016; **61**: 3061-3071 [PMID: 26576555 DOI: 10.1007/s10620-015-3960-x]
7 **Marcellin P**, Zoulim F, Hézode C, Causse X, Roche B, Truchi R, Pauwels A, Ouzan D, Dumortier J, Pageaux GP, Bourlière M, Riachi G, Zarski JP, Cadranel JF, Tilliet V, Stern C, Pétour P, Libert O, Consoli SM, Larrey D. Effectiveness and Safety of Tenofovir Disoproxil Fumarate in Chronic Hepatitis B: A 3-Year, Prospective, Real-World Study in France. *Dig Dis Sci* 2016; **61**: 3072-3083 [PMID: 26821154 DOI: 10.1007/s10620-015-4027-8]
8 **Han Y**, Zeng A, Liao H, Liu Y, Chen Y, Ding H. The efficacy and safety comparison between tenofovir and entecavir in treatment of chronic hepatitis B and HBV related cirrhosis: A systematic review and Meta-analysis. *Int Immunopharmacol* 2017; **42**: 168-175 [PMID: 27915131 DOI: 10.1016/j.intimp.2016.11.022]
9 **Lok AS**, McMahon BJ, Brown RS, Wong JB, Ahmed AT, Farah W, Almasri J, Alahdab F, Benkhadra K, Mouchli MA, Singh S, Mohamed EA, Abu Dabrh AM, Prokop LJ, Wang Z, Murad MH, Mohammed K. Antiviral therapy for chronic hepatitis B viral infection in adults: A systematic review and meta-analysis. *Hepatology* 2016; **63**: 284-306 [PMID: 26566246 DOI: 10.1002/hep.28280]
10 **Chan HL**, Shaikh J, Gupta S, Hamed K. Renal Function in Nucleos(t)ide Analog-Treated Patients With Chronic Hepatitis B: A Systematic Literature Review and Network Meta-Analysis. *Adv Ther* 2016; **33**: 862-875 [PMID: 27146675 DOI: 10.1007/s12325-016-0337-2]
11 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ* 2009; **339**: b2535 [PMID: 19622551 DOI: 10.1136/bmj.b2535]
12 **Stroup DF**, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-

analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000; **283**: 2008-2012 [PMID: 10789670 DOI: 10.1001/jama.283.15.2008]
13 **Huang X**, Lin J, Demner-Fushman D. Evaluation of PICO as a knowledge representation for clinical questions. *AMIA Annu Symp Proc* 2006; 359-363 [PMID: 17238363]
14 **Ha NB**, Ku K, Ha NB, Chaung KT, Trinh HN, Nguyen MH. Renal Function in Chronic Hepatitis B Patients Treated With Tenofovir Disoproxil Fumarate or Entecavir Monotherapy: A Matched Case-Cohort Study. *J Clin Gastroenterol* 2015; **49**: 873-877 [PMID: 25856383 DOI: 10.1097/MCG.0000000000000325]
15 **Lee SW**, Kwon JH, Lee J, Han NI, Kim YW, Nam SW, Chung KW, Jang JW. Comparison of Efficacy between Tenofovir and Entecavir in Treatment Naive Chronic hepatitis B Patients. *Korean Association for the Study of the Liver* 2015; **2015**: 108
16 **Yu HM**, Kwon SY, Kim J, Chung HA, Kwon SW, Jeong TG, An SH, Jeong GW, Yun SU, Min JK, Kim JH, Choe WH. Virologic response and safety of tenofovir versus entecavir in treatment-naïve chronic Hepatitis B patients. *Saudi J Gastroenterol* 2015; **21**: 146-151 [PMID: 26021773 DOI: 10.4103/1319-3767.157558]
17 **Julian PTH**, Sally G. Cochrane handbook for systematic reviews of interventions. John Wiley & Sons, 2008.
18 **Lo CK**, Mertz D, Loeb M. Newcastle-Ottawa Scale: comparing reviewers' to authors' assessments. *BMC Med Res Methodol* 2014; **14**: 45 [PMID: 24690082 DOI: 10.1186/1471-2288-14-45]
19 **Schünemann HBJ**, Brozek J, Guyatt G, Oxman A. GRADE handbook for grading quality of evidence and strength of recommendations. The GRADE Working Group, 2013. Available from: https://gdt.gradepro.org/app/handbook/handbook.html
20 **Park J**, Jung KS, Lee HW, Kim BK, Kim SU, Kim DY, Ahn SH, Han KH, Park JY. Effects of Entecavir and Tenofovir on Renal Function in Patients with Hepatitis B Virus-Related Compensated and Decompensated Cirrhosis. *Gut Liver* 2017; **11**: 828-834 [PMID: 28651305 DOI: 10.5009/gnl16484]
21 **Tsai MC**, Chen CH, Tseng PL, Hung CH, Chiu KW, Chang KC, Yen YH, Lin MT, Hu TH. Does Nucleos(t)ide Analogues Treatment Affect Renal Function in Chronic Hepatitis B Patients Who Have Already Decreased eGFR? A Longitudinal Study. *PLoS One* 2016; **11**: e0149761 [PMID: 26964034 DOI: 10.1371/journal.pone.0149761]
22 **López Centeno B**, Collado Borrell R, Pérez Encinas M, Gutiérrez García ML, Sanmartin Fenollera P. Comparison of the effectiveness and renal safety of tenofovir versus entecavir in patients with chronic hepatitis B. *Farm Hosp* 2016; **40**: 279-286 [PMID: 27571496 DOI: 10.7399/fh.2016.40.4.10492]
23 **Koksal AR**, Alkim H, Boga S, Iyisoy MS, Sen I, Tekin Neijmann S, Alkim C. Value of Cystatin C-Based e-GFR Measurements to Predict Long-Term Tenofovir Nephrotoxicity in Patients With Hepatitis B. *Am J Ther* 2019; **26**: e25-e31 [PMID: 27753647 DOI: 10.1097/MJT.0000000000000518]
24 **Koklu S**, Gulsen MT, Tuna Y, Koklu H, Yuksel O, Demir M, Guner R, Dogan Z, Kucukazman M, Poyrazoglu OK, Biyik M, Ozturk NA, Aydogan T, Coban S, Kocaman O, Sapmaz F, Gokturk SH, Karaca C, Demirezer A, Tanoglu A, Yildirim B, Altinbas A, Atak BM, Cosar AM, Alkan E; Other collaborators. Differences in nephrotoxicity risk and renal effects among anti-viral therapies against hepatitis B. *Aliment Pharmacol Ther* 2015; **41**: 310-319 [PMID: 25982037 DOI: 10.1111/apt.13036]
25 **Idilman R**, Gunsar F, Koruk M, Keskin O, Meral CE, Gulsen M, Elhan AH, Akarca US, Yurdaydin C. Long-term entecavir or tenofovir disoproxil fumarate therapy in treatment-naïve chronic hepatitis B patients in the real-world setting. *J Viral Hepat* 2015; **22**: 504-510 [PMID: 25431108 DOI: 10.1111/jvh.12358]
26 **Buti M**, Tsai N, Petersen J, Flisiak R, Gurel S, Krastev Z, Aguilar Schall R, Flaherty JF, Martins EB, Charuworn P, Kitrinos KM, Subramanian GM, Gane E, Marcellin P. Seven-year efficacy and safety of treatment with tenofovir disoproxil fumarate for chronic hepatitis B virus infection. *Dig Dis Sci* 2015; **60**: 1457-1464 [PMID: 25532501 DOI: 10.1007/s10620-014-3486-7]
27 **Sriprayoon T**, Mahidol C, Ungtrakul T, Chun-On P, Soonklang K, Pongpun W, Laohapand C, Dechma J, Pothijaroen C, Auewarakul C, Tanwandee T. Efficacy and safety of entecavir versus tenofovir treatment in chronic hepatitis B patients: A randomized controlled trial. *Hepatol Res* 2017; **47**: E161-E168 [PMID: 27176630 DOI: 10.1111/hepr.12743]
28 **Marcellin P**, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, Washington MK, Germanidis G, Flaherty JF, Aguilar Schall R, Bornstein JD, Kitrinos KM, Subramanian GM, McHutchison JG, Heathcote EJ. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet* 2013; **381**: 468-475 [PMID: 23234725 DOI: 10.1016/S0140-6736(12)61425-1]
29 **Trinh S**, Le AK, Chang ET, Hoang J, Jeong D, Chung M, Lee MH, Wang U, Henry L, Cheung R, Nguyen MH. Changes in Renal Function in Patients With Chronic HBV Infection Treated With Tenofovir Disoproxil Fumarate vs Entecavir. *Clin Gastroenterol Hepatol* 2019; **17**: 948-956.e1 [PMID: 30130625 DOI: 10.1016/j.cgh.2018.08.037]
30 **Wong GL**, Chan HL, Tse YK, Yip TC, Lam KL, Lui GC, Szeto CC, Wong VW. Chronic kidney disease progression in patients with chronic hepatitis B on tenofovir, entecavir, or no treatment. *Aliment Pharmacol Ther* 2018; **48**: 984-992 [PMID: 30125952 DOI: 10.1111/apt.14945]

Published By Baishideng Publishing Group Inc
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: bpgoffice@wjgnet.com
Help Desk:http://www.f6publishing.com/helpdesk
http://www.wjgnet.com