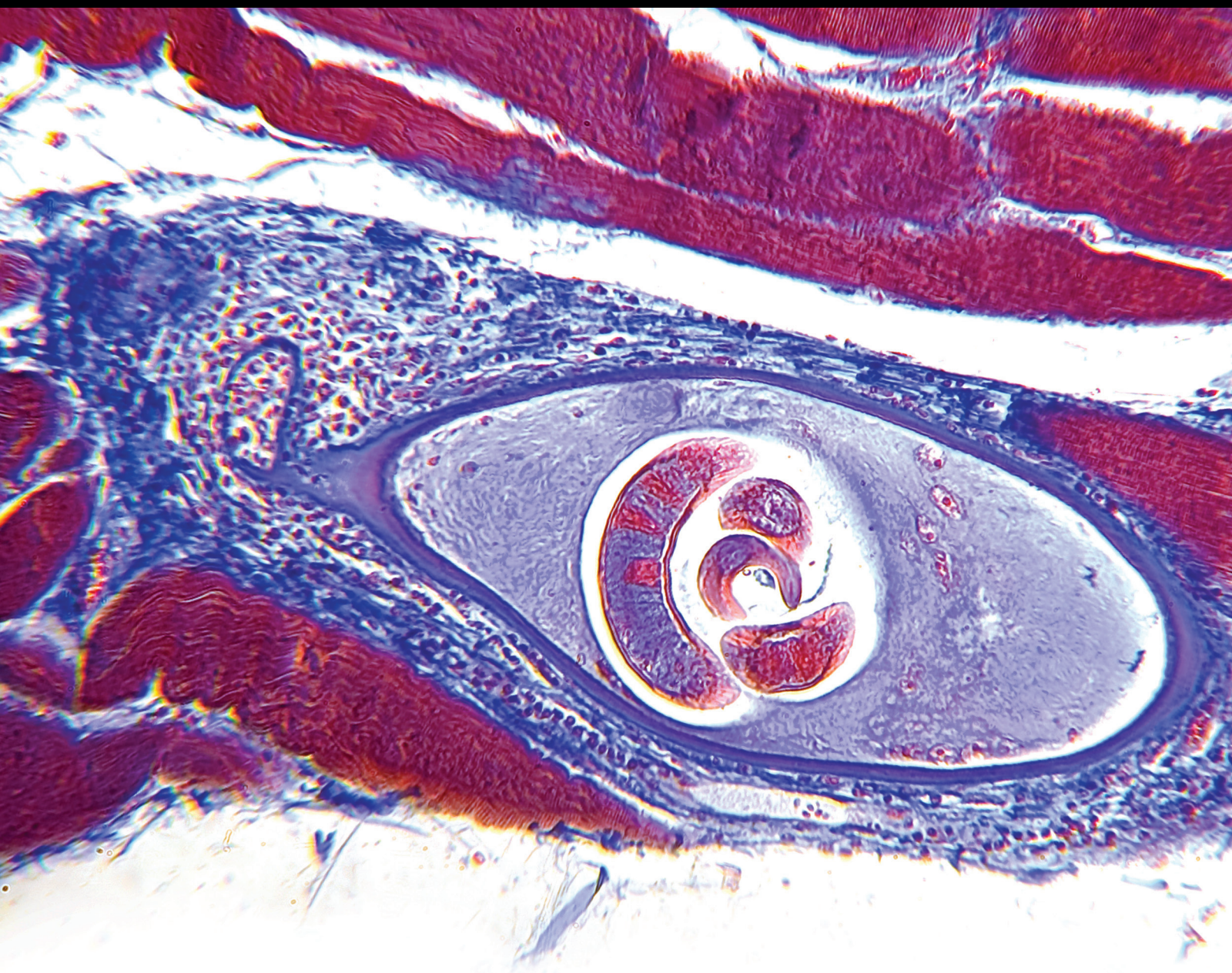
Gastroenterology Research and Practice

Advanced Studies in Clinical and Experimental Research in Gastroenterology

Guest Editors: Ilja Tacheci, Jithinraj Edakkanambeth Varayil, Zuzana Zelinkova, Marcela Kopacova, and Miroslav Zavoral



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Editorial Board

Contents

Advanced Studies in Clinical and Experimental Research in Gastroenterology
Ilja Tacheci, Jithinraj Edakkanambeth Varayil, Zuzana Zelinkova, Marcela Kopacova, and Miroslav Zavoral
Volume 2016, Article ID 4253143, 2 pages

Lower Viral Response to Pegylated Interferon Alpha 2a Treatment of Chronic Hepatitis B in Roma People in Eastern Slovakia
Sylvia Drazilova, Martin Janicko, Pavol Kristian, Ivan Schreter, Branislav Kucinsky, Marek Kozlej, Ivana Hockickova, and Peter Jarcuska
Volume 2016, Article ID 8682494, 6 pages

Surgical Anatomy of the Gastrointestinal Tract and Its Vasculature in the Laboratory Rat
Katarína Vdoviaková, Eva Petrovová, Marcela Maloveská, Lenka Krešáková, Jana Teleky, Mario Zefanias Joao Elias, and Darina Petrášová
Volume 2016, Article ID 2632368, 11 pages

Serum Liver Fibrosis Markers for Predicting the Presence of Gastroesophageal Varices in Liver Cirrhosis: A Retrospective Cross-Sectional Study
Xingshun Qi, Hongyu Li, Jiang Chen, Chunlian Xia, Ying Peng, Junna Dai, Yue Hou, Han Deng, Jing Li, and Xiaozhong Guo
Volume 2015, Article ID 274534, 6 pages

Assessing the Effect of Leptin on Liver Damage in Case of Hepatic Injury Associated with Paracetamol Poisoning
Murat Polat, Serkan Cerrah, Bulent Albayrak, Serkan Ipek, Mahmut Arabul, Fatih Aslan, and Omer Yilmaz
Volume 2015, Article ID 357360, 8 pages

ATAD2 Overexpression Identifies Colorectal Cancer Patients with Poor Prognosis and Drives Proliferation of Cancer Cells
Yang Luo, Guang-Yao Ye, Shao-Lan Qin, Min-Hao Yu, Yi-Fei Mu, and Ming Zhong
Volume 2015, Article ID 936564, 8 pages

Prognostic Value of Metastatic No.8p LNs in Patients with Gastric Cancer
Dong-Jiao Guo, Kun Yang, Wei-Han Zhang, Xiao-Long Chen, Xin-Zu Chen, Bo Zhang, Zong-Guang Zhou, and Jian-Kun Hu
Volume 2015, Article ID 937682, 7 pages

A Randomized Controlled Trial Evaluating a Low-Volume PEG Solution Plus Ascorbic Acid versus Standard PEG Solution in Bowel Preparation for Colonoscopy
Masahiro Tajika, Tsutomu Tanaka, Makoto Ishihara, Nobumasa Mizuno, Kazuo Hara, Susumu Hijioka, Hiroshi Imaoka, Takamitsu Sato, Tatsuji Yogi, Hideharu Tsutsumi, Toshihisa Fujiyoshi, Nobuhiro Hieda, Nozomi Okuno, Tsukasa Yoshida, Vikram Bhatia, Yasushi Yatabe, Kenji Yamao, and Yasumasa Niwa
Volume 2015, Article ID 326581, 12 pages

The Prevalence of Gastric Intestinal Metaplasia and Distribution of *Helicobacter pylori* Infection, Atrophy, Dysplasia, and Cancer in Its Subtypes
Sehmus Olmez, Mehmet Aslan, Remzi Erten, Suleyman Sayar, and Irfan Bayram
Volume 2015, Article ID 434039, 6 pages

Anti-Inflammation Property of *Syzygium cumini* (L.) Skeels on Indomethacin-Induced Acute Gastric Ulceration
Lanchakon Chanudom and Jitbanjong Tangpong
Volume 2015, Article ID 343642, 12 pages

Molecular Features and Methylation Status in Early Onset (≤40 Years) Colorectal Cancer: A Population Based, Case-Control Study
Giulia Magnani, Daniela Furlan, Nora Sahnane, Luca Reggiani Bonetti, Federica Domati, and Monica Pedroni
Volume 2015, Article ID 132190, 10 pages

Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2016, Article ID 4253143, 2 pages
http://dx.doi.org/10.1155/2016/4253143

Editorial

Advanced Studies in Clinical and Experimental Research in Gastroenterology

Ilja Tacheci,[1] Jithinraj Edakkanambeth Varayil,[2] Zuzana Zelinkova,[3] Marcela Kopacova,[1] and Miroslav Zavoral[4]

[1] *2nd Department of Internal Medicine-Gastroenterology, Charles University in Praha, Faculty of Medicine at Hradec Kralove, University Teaching Hospital, Sokolska 581, 500 05 Hradec Kralove, Czech Republic*
[2] *Division of General Internal Medicine, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN 55905, USA*
[3] *5th Department of Internal Medicine, Comenius University in Bratislava, Faculty of Medicine in Bratislava, University Teaching Hospital, Ruzinovska 6, 826 06 Bratislava, Slovakia*
[4] *Department of Internal Medicine, First Faculty of Medicine of Charles University and Military University Hospital, U Vojenské nemocnice 1200, 169 02 Prague, Czech Republic*

Correspondence should be addressed to Ilja Tacheci; ilja.tacheci@fnhk.cz

Received 1 November 2015; Accepted 3 November 2015

Current research in gastroenterology and hepatology (both clinical and experimental) made great and rapid progress in new methods, in biomedical technology, and last but not least in practical application of basic scientific achievements during the last decade.

Original research articles focusing on new, advanced studies in clinical and experimental research in gastroenterology and hepatology that have a potential to influence our daily practice and further clinical and experimental research were sent to this journal to be published by many leading gastroenterology specialists.

We bring ten articles in this special issue focused on the most interesting topics in gastroenterology and hepatology research. One of the leading areas of advanced studies in gastroenterology is cancer research. Colorectal cancer is one of the most common malignancies worldwide in terms of incidence and mortality. Although clear progress in both diagnosis and treatment has been achieved, efficacy of surgery and chemotherapy remains unsatisfactory due to late diagnosis. A possible new prognostic marker and therapeutic target in this disease is discussed in the article "ATAD2 Overexpression Identifies Colorectal Cancer Patients with Poor Prognosis and Drives Proliferation of Cancer Cells." The aim of another article dealing with the colorectal cancer "Molecular Features and Methylation Status in Early Onset (≤40 Years) Colorectal Cancer: A Population Based, Case-Control Study" was to define the frequency of known hereditary colorectal syndromes and to characterise genetic and epigenetic features of early nonhereditary tumours in order to elucidate possible pathogenetic mechanisms. Colonoscopy plays an important role in colorectal cancer screening in many countries worldwide. Quality of colonic cleansing achieved is crucial for good quality of colonoscopy screening. Possible improvements in the field of low volume colonoscopy cleansing were described in the article "A Randomized Controlled Trial Evaluating a Low-Volume PEG Solution Plus Ascorbic Acid versus Standard PEG Solution in Bowel Preparation for Colonoscopy." Second malignancy, which is discussed in a special issue in more detail, is the third most common cause of cancer-related death in the world: the gastric cancer. In the article "The Prevalence of Gastric Intestinal Metaplasia and Distribution of *Helicobacter pylori* Infection, Atrophy, Dysplasia, and Cancer in Its Subtypes," the prevalence of the precancerous gastric lesions (intestinal metaplasia especially) in a large cohort of patients within the region with a high risk of gastric cancer was described. The metastatic involvement of the posterior lymph nodes along the common hepatic artery (the extra-regional lymph nodes

called No.8p) was identified as a prognostic factor in the retrospective study: "Prognostic Value of Metastatic No.8p LNs in Patients with Gastric Cancer."

The research based on the use of experimental animals still plays an important role in gastroenterology and the rat is one of the most extensively used experimental animal models.

The study "Surgical Anatomy of the Gastrointestinal Tract and Its Vasculature in the Laboratory Rat" investigated the functional anatomy and vasculature of the stomach, liver, and intestine in the laboratory rat and compared it with human morphology.

Nonsteroidal anti-inflammatory drug-induced gastropathy and ulcers represent an important complication related to one of the most commonly used drugs worldwide. Possible mucosal protective effect of *Syzygium cumini* (L.) Skeels aqueous extract in experimental mice model of indomethacin-induced gastric damage was proven in article "Anti-Inflammation Property of *Syzygium cumini* (L.) Skeels on Indomethacin-Induced Acute Gastric Ulceration."

Research in hepatology is represented in three articles: "Assessing the Effect of Leptin on Liver Damage in Case of Hepatic Injury Associated with Paracetamol Poisoning," "Lower Viral Response to Pegylated Interferon Alpha 2a Treatment of Chronic Hepatitis B in Roma People in Eastern Slovakia," and "Serum Liver Fibrosis Markers for Predicting the Presence of Gastroesophageal Varices in Liver Cirrhosis: A Retrospective Cross-Sectional Study."

The special issue cannot be a complete overview of research in the field of gastroenterology and hepatology. However, we believe that it is an interesting look at this area and provides a preview with an important impact on clinical medicine.

Ilja Tacheci
Jithinraj Edakkanambeth Varayil
Zuzana Zelinkova
Marcela Kopacova
Miroslav Zavoral

Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2016, Article ID 8682494, 6 pages
http://dx.doi.org/10.1155/2016/8682494

Clinical Study

Lower Viral Response to Pegylated Interferon Alpha 2a Treatment of Chronic Hepatitis B in Roma People in Eastern Slovakia

Sylvia Drazilova,[1] Martin Janicko,[2] Pavol Kristian,[3] Ivan Schreter,[3] Branislav Kucinsky,[2] Marek Kozlej,[2] Ivana Hockickova,[3] and Peter Jarcuska[2]

[1]*Department of Internal Medicine, Poprad Hospital, Banícka 28, 05845 Poprad, Slovakia*
[2]*1st Department of Internal Medicine, Medical Faculty of Pavol Jozef Šafárik University in Košice, Trieda SNP 1, 04001 Košice, Slovakia*
[3]*Department of Infectology and Travel Medicine, Medical Faculty of Pavol Jozef Šafárik University in Košice, Trieda SNP 1, 04001 Košice, Slovakia*

Correspondence should be addressed to Peter Jarcuska; petjarc@yahoo.com

Received 14 March 2015; Accepted 18 August 2015

Academic Editor: Ilja Tacheci

Aim. To evaluate the compliance and virological response to pegylated interferon alpha 2a treatment of chronic hepatitis B in Roma population compared to majority Caucasian population in Slovakia. *Methods*. Retrospective evaluation of a cohort of all Roma patients treated with pegylated interferon alpha 2a from 2007 to 2013 in 3 centers for treatment of chronic viral hepatitis B. The Study included 43 Roma patients with chronic viral hepatitis B and randomly selected control group. Treatment duration was 48 weeks. Viral response was evaluated after 24 weeks, at the end of treatment, and 24 weeks after the end of treatment. *Results*. Complete treatment course was finished by 79.1% of Roma patients compared to all patients from the control group ($p = 0.0009$). There was a tendency toward lower viral response rate in Roma at all time points; however significant difference was only at end of treatment viral response (51.2% Roma versus 81.4% majority, $p = 0.003$). We also did not find significant difference at the rate of HBsAg loss. *Conclusion*. Roma patients with chronic hepatitis B have significantly worse compliance to treatment with pegylated interferon and they have significantly lower rate of end of treatment viral response.

1. Introduction

Hepatitis B virus infection remains to be a serious healthcare problem. Chronic viral hepatitis is recognized to be the leading cause of liver cirrhosis worldwide [1]. Around 350–400 million of people worldwide are infected at one time with hepatitis B virus, but more than two billion people are infected with hepatitis B virus during their lifetime [2]. Chronic hepatitis B (CHB) can result in liver cirrhosis and hepatocellular carcinoma, which can occur even in inactive carriers of hepatitis B virus without liver cirrhosis [3]. Every year more than one million people die due to liver failure as a direct consequence of hepatitis B infection [4]. One of CHB treatment options remains pegylated interferon. This therapy not only leads to the improvement of biochemical, virological, and histological findings but also causes twofold reduction of liver cirrhosis risk and 5-fold reduction of hepatocellular carcinoma risk in the average time horizon of 6.8 years [5].

Roma population, due to its peculiarities, poses a significant challenge to the healthcare system. It is estimated that almost two million Roma people live in Central and Eastern Europe alone [6]. Although, due to the significant difference between the official demographic statistics and the estimated count, it is not possible to determine the exact Roma population count, it is reasonable to assume that it represents a substantial population size. Roma population in Eastern Slovakia tends to segregate from the majority population in closed settlements. Their way of living combined with the nonvaccination creates an environment with high risk for hepatitis B infection. In fact, in our previous studies we have determined that the prevalence of hepatitis B in these communities is as high as 12.5% [7]. In this study we wanted to evaluate the treatment effectiveness

Table 1: Baseline demographic and virological parameters of the study cohort.

	Roma	Non-Roma	p
Count	43	46	
Age (years ± SD)	33 ± 8.3	35 ± 7.5	0.081
Women	11 (25.6%)	13 (28.3%)	0.680
HBeAg posit	9 (20.9%)	4 (8.7%)	0.093
HBV DNA IU/mL (median(IQR))	40900 (28900–588000)	27550 (3300–223367)	0.025
HBV DNA < 2000 IU/mL	3 (9.3%)	7 (15.2%)	0.219
ALT > 2 ULN	22 (51.2%)	21 (45.7%)	0.603
Staging (Metavir)	1.64 ± 1.07	1.34 ± 1.04	0.382
Grading (Metavir)	1.91 ± 0.87	2.0 ± 0.76	0.795
Cirrhosis	3 (7.0%)	5 (10.9%)	0.521

ALT: alanine aminotransferase.

and compliance in this population and compare it with the majority.

2. Methods

This retrospective observational cohort study included all Roma patients with CHB treated with pegylated interferon alpha 2a (PEG IFN) from 2007 to 2013 in three centers for treatment of viral hepatitis (1st Department of Internal Medicine and Department of Infectious Diseases and Travel Medicine, Pavol Jozef Šafárik University in Košice, and University Hospital of L. Pasteur in Košice and Department of Internal Medicine, Poprad Hospital). All treatment was reimbursed by the national healthcare system and was completely free of charge for the recipients. All administrative background was handled by the prescribing physician. Altogether 43 Roma patients were included. Control group consisted of 46 randomly chosen patients from non-Roma (majority Caucasian) population treated with PEG IFN for CHB that were matched by gender. Age matching was impossible because of the lack of patients with appropriate age in the control group. Study was performed in accordance with principles of the Declaration of Helsinki. Institutional Ethics Committee of Pavol Jozef Šafárik University in Košice waived the need for informed consent based on the retrospective nature of the study.

General indications for CHB treatment with PEG IFN in these three centers were observed:

(i) elevated alanine aminotransferase (ALT) activity in the previous 6 months,

(ii) replication of hepatitis B virus.

Patients were treated with 180 mcg of PEG IFN (Pegasys, Roche, Switzerland) once a week for 48 weeks. HBsAg, HBeAg, and anti-HBs antibodies were tested by Enzygnost (Siemens, Germany) before start of the treatment, at the end of the treatment (M12), and 6 months after the end of the treatment (M18). HBV DNA was tested quantitatively before start of the treatment, 6 months after the start of treatment (M6), at the end of the treatment (M12), and 6 months after the end of the treatment (M18) by Cobas AmpliPrep/Cobas 2.0 (Roche, Switzerland) with the lower limit of detection at 20 IU/mL. Routine biochemical analyses were performed by Siemens Advia 2400 autoanalyzers (Siemens, Germany). Liver cirrhosis was evaluated either histologically or indirectly by liver morphology and clinical signs.

Virological response was present if patients achieved HBV DNA levels <2000 IU/mL at any time point; sustained virological response was present if patients had HBV DNA < 2000 IU/mL 6 months after the end of treatment (EoT). Complete virological response was defined by undetectable HBV DNA and loss of HBsAg [8].

Values are reported as mean ± standard deviation or absolute and relative counts. HBV DNA was transformed using decadic logarithm and reported as median (interquartile range). Continuous variables were compared using Mann-Whitney test. Categorical variables were compared by chi-squared test or Fisher's exact test. Adjusted odds ratios were calculated by multivariate logistic regression. Analyses of virological response for the different time points were performed for per-protocol population. Due to large dropout of Roma patients also real-world effectiveness, which included dropout Roma patients as nonrespondents, was calculated.

3. Results

Out of all 43 enrolled Roma patients, 39 remained in the study at M6, 32 remained at EoT (M12), and 29 returned also 6 months after the end of the treatment (M18) for followup. On the other hand, all patients from the control group completed the treatment and 34 out of 46 were available for followup at M18.

Baseline parameters of the study cohort are reported in Tables 1 and 2. Roma patients had significantly higher HBV DNA levels (Figure 1), but we did not find any difference in the HBeAg positivity, significantly increased ALT, HBV DNA < 2000 IU/mL, or prevalence of liver cirrhosis between Roma and control group. Biopsy was performed in half of the participants, but no difference in average METAVIR score was detected. Also no difference was found in selected biochemical and hematological tests, except the levels of HDL cholesterol (Table 2).

Roma patients had significantly worse compliance to the treatment. All majority patients completed the whole

Table 2: Baseline biochemical and hematological parameters of the study cohort.

	Roma (mean ± SD)	n	Non-Roma (mean ± SD)	n	p
ALT (μkat/L)	1.52 ± 1.47	43	1.62 ± 1.22	46	0.703
GGT (μkat/L)	0.90 ± 0.64	43	1.06 ± 1.15	46	0.977
TC (mmol/L)	4.90 ± 0.90	43	5.10 ± 0.95	43	0.478
LDL-C (mmol/L)	3.00 ± 0.60	22	3.14 ± 0.87	35	0.605
TAG (mmol/L)	2.37 ± 4.72	27	1.26 ± 0.69	43	0.167
HDL-C (mmol/L)	1.18 ± 0.30	25	1.47 ± 0.36	44	0.003
Bilirubin (μmol/L)	11.95 ± 5.77	43	13.70 ± 6.75	46	0.349
Albumin (g/L)	46.91 ± 4.44	43	45.24 ± 3.55	46	0.176
Platelets (10^9/L)	201.40 ± 60.05	43	198.82 ± 65.29	45	0.754
WBC (10^9/L)	6.43 ± 2.28	43	6.03 ± 1.46	45	0.628

ALT: alanine aminotransferase, GGT: gamma-glutamyl transferase, TC: total cholesterol, LDL-C: low density lipoproteins, TAG: triacylglycerols, HDL-C: high density lipoproteins, and WBC: white blood cells.

Table 3: HBV DNA during treatment and followup, per-protocol data.

	Roma		Non-Roma		p
M6 HBV DNA <2000 IU/mL	26/39	66.7%	37/44	84.1%	0.06
M6 HBV DNA negative	3/39	7.7%	7/44	15.9%	0.172
M12 HBV DNA <2000 IU/mL	22/32	68.8%	37/46	80.4%	0.237
M12 HBV DNA negative	3/32	9.4%	9/46	19.6%	0.220
M18 HBV DNA <2000 IU/mL	13/29	44.8%	19/34	55.9%	0.382
M18 HBV DNA negative	0/29	0%	3/34	8.8%	0.167

M6: 6 months after the start of the treatment, M12: end of treatment, and M18: 6 months after the end of the treatment.

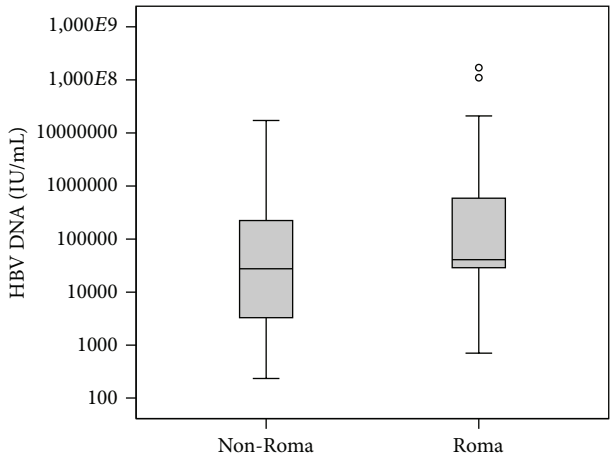

Figure 1: HBV DNA before treatment in Roma and majority (non-Roma) population.

treatment course compared to only 79.1% of Roma patients ($p = 0.0009$). There was also a tendency toward higher loss of followup among Roma patients at M18 compared to control group (32.6% versus 26.1%; $p = 0.502$) (Table 5).

There was a tendency toward lower virological response in Roma patients in all of the specified time points; however per-protocol analysis showed no difference between Roma and control groups in the achievement of virological response at M6, M12, or M18 as well as in the rate of undetectable HBV DNA (Table 3). However, real-world EoT response was significantly more frequent in non-Roma patients compared to Roma patients (80.4% versus 51.2%) (Table 4). This was true even after adjustment for age and sex (OR for Roma 0.148; 95% CI 0.047–0.467). No significant difference was present in the prevalence of virological response at M6 or M18, or the rate of HBV DNA negativization in any of the time points (Tables 3 and 4). All patients who achieved virological response (HBV DNA < 2000 IU/mL) had ALT within normal range at M12 and M18.

There was also a tendency toward lower rates of HBsAg loss and anti-HBs seroconversion in Roma people compared with control group at all time points. However absolute occurrences of both HBsAg loss and anti-HBs seroconversion were very low (less than 4% in Roma and less than 10% in non-Roma population) and the differences were statistically insignificant.

Out of 9 HBeAg positive Roma patients 6 completed the treatment. Three patients lost HBeAg and gained anti-HBe antibodies which lasted up to 6 months after EoT. Two of these patients had HBV DNA at EoT and 6 months after the EoT less than 2000 IU/mL. In the control group only one patient underwent HBeAg seroconversion, but this patient had HBV DNA over 2000 IU/mL at all time points. No patient from both groups who did not lose HBeAg had HBV DNA less than 2000 IU/mL.

In the course of the treatment no serious adverse events or any other important medical events were recorded. After EoT 2 patients from the majority population died, one due to liver failure and one due to abdominal aneurysm dissection.

Table 4: HBV DNA during treatment and followup, real-world cohort.

	Roma		Non-Roma		p
M6 HBV DNA <2000 IU/mL	30/43	69.8%	38/46	82.6%	0.154
M6 HBV DNA negative	3/43	7.0%	7/46	15.2%	0.318
M12 HBV DNA <2000 IU/mL	22/43	51.2%	37/46	80.4%	0.003
M12 HBV DNA negative	3/43	7.0%	9/46	19.6%	0.082
M18 HBV DNA <2000 IU/mL	13/43	30.2%	19/46	41.3%	0.277
M18 HBV DNA negative	0/43	0%	3/46	6.5%	0.242

M6: 6 months after the start of the treatment, M12: end of treatment, and M18: 6 months after the end of the treatment.

Table 5: Compliance.

	Roma		Non-Roma		p
Uncompleted treatment (48 weeks)	9/43	20.9%	0/46	0%	0.0009
Loss to followup at M18	14/43	32.6%	12/46	26.1%	0.502

4. Discussion

Chronic hepatitis B can be treated by PEG IFN or nucleot(s)ide analogues (NUC). Interferon based treatment is, compared to NUC treatment, temporary [8]. Based on the published data, 48-week treatment course with 180 mcg PEG IFN once weekly leads to anti-HBe antibody formation in 27% of patients at EoT and 32% of patients at week 24 after EoT with PEG IFN. Decrease of HBV DNA under 100 000 copies/mL occurs in 52% of patients at EoT and 32% of patients 24 weeks after EoT. Decrease of HBV DNA under 400 copies/mL occurs in 25% and 14% of patients at EoT and 24 weeks after EoT, respectively. Loss of HBsAg is sporadic. At week 72 after EoT occurs in 3% of patients, most of these patients have genotype A [9].

In the multicentric study which enrolled HBeAg negative patients treated with PEG IFN in the same dose and duration, HBV DNA decreased under 20 000 copies/mL in 81% at EoT and in 43% it remained under 20 000 copies/mL 24 weeks after EoT. Only in 63% of patients HBV DNA decreased under 400 copies/mL at EoT and in 19% it remained under 400 copies/mL at month 6 after EoT. HBsAg loss occurred in less than 4% of patients [10].

Non-Roma patients in our study achieved similar results; however in Roma patients the results were marginally worse. In every time point there was a trend toward worse outcome of the treatment in Roma population, which achieved statistical significance when the virological response at EoT was evaluated. To our knowledge no study was published in the current literature that would evaluate the efficacy of PEG IFN treatment in the Roma population; therefore we have no data for comparison. Multiple host related factors influence the PEG IFN treatment efficacy. It has been shown that Asian race and genotype A favor the HBsAg seroconversion [10]. Conversely, E genotype that is more prevalent on the African continent is associated with poorer PEG IFN treatment efficacy [11]. Studies that would identify other socioeconomic risk factors for poor PEG IFN treatment efficacy are lacking.

The reasons for worse efficacy of treatment in this particular population are not known. One of the possible causes of this phenomenon is the lower compliance to treatment and lower rate of successfully completed treatment by Roma patients (21% dropout compared to 0% dropout in the control group). Lower socioeconomic standard and other factors associated with segregation in marginalized communities are also possible causes of lower treatment efficacy [12]. Significant portion of Roma patients in our study came from such settlements. In a recent study these people reported worse healthcare availability, higher distrust to medical personnel, and higher anxiety of medical examination. Moreover, they tend to prefer their own alternative treatments compared to the majority population [13].

PEG IFN treatment adherence is not optimal. In the prospective randomized controlled trial by Janssen et al. only 84% completed the treatment (12 are withdrawn for misconduct and 11 discontinued therapy early because of side effects) and further 7 (5.6%) were lost to followup [14]. Real-life studies with posttreatment followup for at least 6 months documented the loss of patients ranging from 3.4% [15] to 20% [16]. In our study, the loss of followup at M18 was 26% in majority population and 37% in Roma population. In a study focused on the long-term NUC treatment of hepatitis B poorer adherence was significantly associated with female sex, younger age, and lower income. This corresponds to our patients because Roma people were significantly younger and poorer.

We can only speculate about other reasons for this reported lower PEG IFN treatment efficacy. The origins of Roma population of Europe have not been confirmed so far, but the prevailing opinion is that they migrated to Europe from India. We therefore performed a search of Web of Science, Medline, and Scopus databases but found no study which evaluated the efficacy of PEG IFN treatment specifically in Indian population. Three small studies reported the efficacy of CHB treatment with recombinant interferon alpha. One randomized study included 20 patients treated for 4 months with recombinant interferon alpha 2b dosed 3 MIU three times weekly for four months. Loss of HBeAg with negative HBV DNA was present in 50% of patients after the end of the treatment and in 65% 12 months after

the end of the treatment. Only three patients lost HBsAg as well. CHB relapse was not documented in any of these patients [17]. Another study reported the results of the same treatment regimen in HBeAg positive patients. Mean time of followup was 2.2 years after EoT. Nine out of 14 patients lost HBeAg (64%) and only one patient lost HBsAg as well [18]. In our study Roma people achieved worse results despite the treatment with PEG IFN for 48 weeks. HBeAg seroconversion was present in 50% of Roma who completed the whole treatment course and only one-third of Roma achieved HBeAg seroconversion as well as HBV DNA under 2000 IU/mL.

Third study from India included only anti-HBe positive patients treated with recombinant interferon alpha 2b in the same regimen as reported in previous two studies. At EoT 13 out of 18 patients were HBV DNA negative; however 7 out of these relapsed in the next 12 months after EoT. Relapse was more frequent in cirrhotic patients [19]. In our study, no Roma patient had negative HBV DNA 6 months after EoT. Decrease of HBV DNA under 2000 IU/mL was detected at EoT (M12) in 76.9% anti-HBe positive Roma patients who completed the full treatment course, but only in 47.8% of patients HBV DNA remained low 6 months after EoT. However it is probable that the treatment population in the three mentioned Indian studies was probably highly selected and the efficacy of interferon based treatment in unselected population would be considerably lower.

Another factor that could influence the virological response to treatment with PEG IFN is virus genotype. PEG IFN treatment is more effective in genotypes A and B compared to genotype C or D; moreover, patients with genotype C or D have more unfavorable course of the disease [20, 21]. The relative geographical prevalence of viral genotypes depends greatly on the migration patterns [22]. The most common HBV genotype in Scandinavia, Germany, Czech Republic, Belgium, Hungary, and Poland is genotype A. On the other hand, in Latvia, Estonia, Spain, Italy, Albania, Croatia, Romania, Serbia, Greece, and Russia, the most common genotype is genotype D [23]. Unfortunately the data about the most prevalent genotype in Slovakia are not known. In the Czech Republic the most common is genotype A (67%), followed by genotype D (28%). Only 3% of patients have genotype B and 2% have genotype C. In Hungary the prevalence of VHB genotypes is very similar, with genotype A being the most common (47%), followed by genotype D (43%) and mixed genotypes in 7% of patients [23]. Roma people however are relatively closed ethnic minority with different origins than the majority population. Therefore, the genotypes most prevalent in these people probably differ from the genotypes in the majority population. The probable place of origin of this minority is North/Northwestern India [24]. Most prevalent genotypes in this region are genotypes A and D (46% and 48%, resp.), followed by patients with mixed genotype. Genotype C in this region is practically nonexistent [25]. The most common genotype in East India is genotype D (75%), followed by genotype A (25%). Genotype C is present in 18% of patients and has a higher risk of hepatocellular carcinoma than other genotypes [22]. We are planning to determine the virus genotypes in this population, which could partially explain the differences in reported treatment efficacy.

Hepatitis B prevalence in Roma population in Slovakia is as high as 12.5% [7] and treatment is very expensive. Improvement of socioeconomic conditions, lifestyle, and poverty among Roma people could help to decrease the burden of the disease in this population [26]. The vaccination against hepatitis B in this high risk population could potentially radically decrease the prevalence of the disease and decrease the risk of progression to liver cirrhosis, liver failure, and hepatocellular carcinoma [4]. Vaccine has been discovered in 1971, but nationwide vaccination of newborns against hepatitis B in Slovakia started in 1998 [27, 28]. The prevalence of hepatitis B in this age category is unknown, but it is supposedly lower than that in older age groups. The people born before 1998 are not vaccinated and they are the main reservoir of the infection in the Roma population. Treatment with PEG IFN or NUCs is expensive and adherence in Roma population is poor, which was demonstrated in our study. Optimal solution from the medical as well as economical point of view would be the vaccination of adult patients who have not come into the contact with hepatitis B.

5. Conclusion

The results of this study have shown that although the PEG IFN treatment of CHB in Roma population is effective, it is less effective than that in the majority population. More research needs to be done to identify the causes of this phenomenon, although observed lower compliance to treatment is one possible explanation. Substantial combined effort is required to effectively decrease the burden of hepatitis B among Roma people.

Abbreviations

CHB: Chronic hepatitis B
EoT: End of treatment
PEG IFN: Pegylated interferon alpha 2a.

Conflict of Interests

S. Drazilova, P. Jarcuska, P. Kristian, and I. Schreter report being on the advisory board and giving lectures for Roche Slovakia. M. Janicko, B. Kucinsky, M. Kozlej, and I. Hockickova declare no conflict of interests.

Authors' Contribution

The authors declare that they contributed equally to the paper and every author fulfills the criteria for authorship.

Acknowledgment

All funding was provided by P. J. Šafárik University in Košice, Slovakia.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2016, Article ID 2632368, 11 pages
http://dx.doi.org/10.1155/2016/2632368

Research Article

Surgical Anatomy of the Gastrointestinal Tract and Its Vasculature in the Laboratory Rat

Katarína Vdoviaková,[1] Eva Petrovová,[1] Marcela Maloveská,[1] Lenka Krešáková,[1] Jana Teleky,[1] Mario Zefanias Joao Elias,[2] and Darina Petrášová[3]

[1]*Department of Anatomy, Histology and Physiology, University of Veterinary Medicine and Pharmacy in Kosice, Komenského 73, 041 81 Kosice, Slovakia*
[2]*Faculty of Veterinary Medicine, Eduardo Mondlane University, P.O. Box 254, 0001 Maputo, Mozambique*
[3]*Laboratory of Research Biomodels, Faculty of Medicine, Pavol Jozef Šafárik University in Kosice, Trieda SNP 1, 04011 Kosice, Slovakia*

Correspondence should be addressed to Katarína Vdoviaková; katarina.vdoviakova@uvlf.sk

Received 13 March 2015; Revised 19 June 2015; Accepted 15 July 2015

Academic Editor: Ilja Tacheci

The aim of this study was to describe and illustrate the morphology of the stomach, liver, intestine, and their vasculature to support the planning of surgical therapeutic methods in abdominal cavity. On adult Wistar rats corrosion casts were prepared from the arterial system and Duracryl Dental and PUR SP were used as a casting medium and was performed macroscopic anatomical dissection of the stomach, liver, and intestine was performed. The rat stomach was a large, semilunar shaped sac with composite lining. On the stomach was very marked fundus, which formed a blind sac (*saccus cecus*). The rat liver was divided into six lobes, but without gall bladder. Intestine of the rat was simple, but cecum had a shape as a stomach. The following variations were observed in the origin of the cranial mesenteric artery. On the corrosion cast specimens we noticed the presence of the anastomosis between middle colic artery (*a. colica media*) and left colic artery (*a. colica sinistra*). We investigated the second anastomosis between middle colic artery and left colic artery. The results of this study reveal that the functional anatomical relationship between the rat stomach, liver and intestine is important for the development of surgical research in human and veterinary medicine.

1. Introduction

The laboratory animals are suitable subjects for many modern experimental and biomedical research including metabolic and immunological studies, tumor and cancer investigation, anatomical, physiological, and biochemical research, and experimental transplantation. Many structures or organs of the body of laboratory animals were studied by many authors, for instance, the mouse [1, 2], hamster [3, 4], and rabbit [5, 6], but some details have not yet been examined. Frequently, the laboratory mammals are also used as animal models for veterinary and human research. The knowledge of anatomical variations is more important for experimental investigation and surgical practice. The investigation of anatomy, comprising the morphology of the vessels in laboratory animals, is nearly united with ischemia and transplantation of the organs.

Nowadays, the laboratory rat is one of the most popular experimental models for the research, because it is easy to handle and inexpensive. The laboratory rat is by far the most used animal model in transplantation of liver and intestine [7, 8] and gastrointestinal tract diseases studies. Monchik and Russell made in 1971 the first experimental transplantation of the intestine in laboratory rats [9]. Compared to humans, the laboratory rats have similar anatomical structure of the body organs.

The aim of this study was to describe and illustrate the morphology of the gastrointestinal tract and its vasculature to support the planning of surgical therapeutic methods in abdominal cavity. The topography of the gastrointestinal organs and variations of the vascularisation are more important in the methods of experimental ischemia and the transplantations.

2. Material and Methods

The experiment was carried out on 20 laboratory rats (*Rattus norvegicus f. domestica*) aged one year, breed Wistar of both sexes (10 females, 10 males), and weighing approximately 350–520 g in the standard breeding condition. Animals were obtained from accredited Laboratory of Research Biomodels, University of P. J. Safarik in Kosice. The experiment on rats was performed with approval of the Ethics Committee of the University of Veterinary Medicine and Pharmacy in Kosice and State Veterinary and Food Institute in Bratislava (number SK P 12004) followed by Slovakian protocols for ethical standards for the use of laboratory animals. The first group of rats (10 animals) was used for the corrosion casts of the arteries and the second group of rats (10 animals) was used for dissection of the gastrointestinal organs.

2.1. The Preparing of the Corrosion Casts Specimens of the Arterial System. Anaesthesia of animals was induced by intraperitoneal injection of sodium thiopental (50 mg/kg, Thiopental Valeant, Valeant Czech Pharma, Czech Republic). Under total anaesthesia we dissected the left ventricle of the heart. We implemented a cannula into the aorta through the left ventricle while the cannula was supported by ligature. A portion of the venous system must be opened to ensure a good distribution of the perfusion medium. The right auricular appendage served this purpose. Vessels were perfused with isotonic saline 0,9% physiological solution at a low flow rate (about 10 mL/min) for 30 s through the left cardiac ventricle. An improved method for the washing out of clotted blood from the vessels was achieved by the addition of 0,05% NaOH (Mikrochem, Slovakia) into the perfusion medium. The perfusion pressure was approximately 200–250 mm Hg (2,6–3,25 m H_2O). The success of the perfusion was indicated by the uniform fading of the tissues seen during the procedure. We mixed the injection media in stechiometric rates. The corrosion casts were prepared with Duracryl Dental resin (Spofa, Dental, Czech Republic) and PUR SP resin (Ústav polymérov, SAV, Slovakia). Suitable colour tone was achieved by addition of 2-3 drops of red (oil, red paint 0). After a proper mixing of all components we applied this mass into the arterial system through the left ventricle of the heart. After vascular casting with the resin is complete, it (and the animal) must not be manipulated for 30 min, after which the casts are submersed in water at a temperature ranging from 40°C to 60°C for a period between 30 min and 24 h for full polymerization of resin [10]. The maceration of the soft tissues was carried out in 2–4% solution of KOH (Mikrochem, Slovakia) at 60–70°C. The maceration took approximately 2-3 days. Prior to the outset of the drying process, the corroded specimens were submersed in water and dried at room temperature. The results were listed in percentages.

2.2. The Macroscopic Anatomical Dissection of the Gastrointestinal Organs. In the second group of rats under total anaesthesia, macroscopic anatomical dissection of the stomach, liver, and intestine was performed and features were compared to humans. The abdominal cavity was opened by the mid-laparotomy, through the abdominal wall in the midline (*linea alba*) from the caudal end of the sternum (*processus xiphoideus*) to the pubic bone (*pecten ossis pubis*). The abdominal wall was cut on both sides, cranially along the last rib and caudally along the inguinal region. A stereostatic microscope (Leica M 320) was used for anatomical dissection, and pictures were taken with a digital camera adapted to the microscope. The latest edition of the Veterinary Anatomic Nomenclature was consulted throughout this study [11].

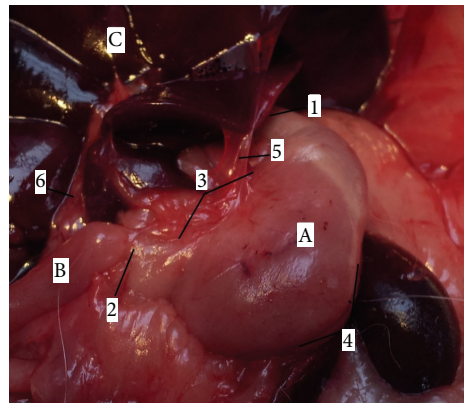

Figure 1: Morphology of rat stomach (*facies parietalis*). 1: *pars cardiaca*, 2: *pars pylorica*, 3: *curvatura ventriculi minor*, 4: *curvatura ventriculi major*, 5: *lig. hepatogastricum*, 6: *ductus hepaticus communis*, A: *ventriculus* (*facies parietalis*), B: *duodenum*, and C: *facies visceralis hepatis*.

3. Results

3.1. Stomach Anatomy. The stomach (*ventriculus*) of rat was situated in the left part of the abdominal cavity, at the level of the last thoracic and first lumbar vertebrae, dorsally to the liver and it was directed transversally. The rat stomach was a large, semilunar shaped sac and weighed between 3,90 and 8,50 g. The stomach tissue represented approximately 1,8% of the total body weight. The left part of the stomach was cardiac part (*pars cardiaca*) and the right was pyloric part (*pars pylorica*). We described two surfaces on the rat stomach. The cranial, parietal surface (*facies parietalis*) was in contact with diaphragm and left abdominal wall. The part of the parietal surface was covered by left lobe of the liver. The caudal, visceral surface (*facies visceralis*) was attached to the intestine. The omentum majus separated jejunum (*jejunum*) and cecum (*cecum*) from the greater omentum (*facies visceralis*) of the stomach. These two surfaces were fused in greater and lesser curvature (*curvature major* and *minor*). The greater curvature was directed caudoventrally. The esophagus entered in the middle of the lesser curvature, which was directed craniodorsally. The stomach had a very marked fundus (*fundus ventriculi*), which formed a distinct craniodorsal blind ventricular sac (*saccus cecus ventriculi*) on the left side, near the cardiac part (Figure 1). Between stomach and abdominal wall adipose pad was situated, which was contributed according to sex into mesorchium (*mesorchium*), mesovarium (*mesovarium*), and mesometrium (*mesometrium*). The rat stomach was joined to visceral surface of the liver by the hepatogastric ligament

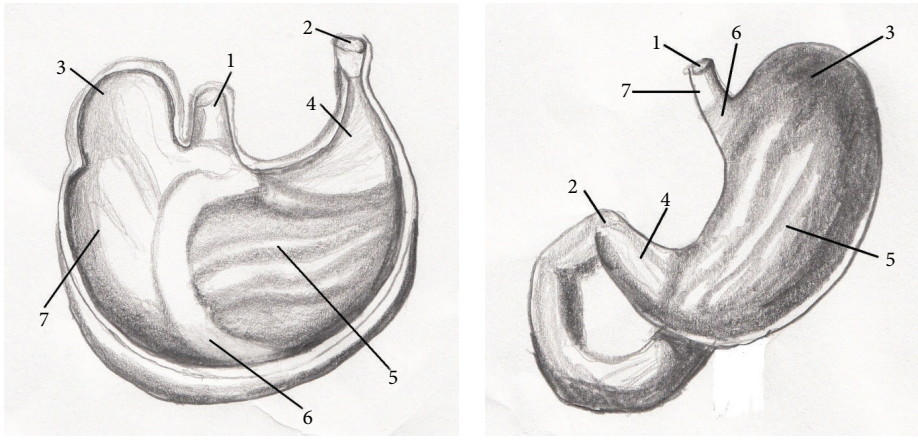

Figure 2: The comparative anatomy of gastric mucous membrane in rat and human. 1: *pars cardiaca*, 2: *pars pylorica*, 3: *fundus ventriculi*, 4: *glandulae pyloricae*, 5: *glandulae gastricae propriae*, 6: *glandulae cardiacae*, 7: *pars nonglandularis*, (a) rat stomach (*facies visceralis*), and (b) human stomach (*paries anterior*).

(*lig. hepatogastricum*). The spleen bordered on the greater curvature of the stomach. These two organs were fused by the gastrosplenic ligament (*lig. gastrolienale*). The tunica mucosa was divided into two parts. The right glandular part (*pars glandularis*) was opaque, muscular, thick walled, and reddish containing the fundic and pyloric regions. This part included gastric glands (cardiac, proper gastric, and pyloric). The cardiac glands were found behind the nonglandular part. There was the narrow strip of cardiac glands (*glandulae cardiacae*) next to the line of transition of the mucous membrane. The pyloric glands were presented only in a zone around the right part of the stomach, around the pylorus (*pylorus*). The surface between these two groups of the glands was covered by the proper gastric glands, in the part of the area of the body of stomach (*corpus ventriculi*). The left, nonglandular part (*pars nonglandularis*) of the stomach was translucent and thin walled (Figure 2). This mucous membrane presented the continuation of the nonglandular mucous membrane of esophagus. This portion was used in the storage and digestion of food.

3.2. Liver Anatomy. The position of rat liver (*hepar*) was in the right side of the abdominal cavity; it was attached to the diaphragm within the rib cage. The liver extended alongside the abdominal wall ventrally beyond the ribs. The rat liver was multilobulated; liver tissue represented approximately 5% of the total body weight and it weighed approximately 13,8 g. The liver had generally two surfaces. The diaphragmatic (Figure 3), convex surface (*facies diaphragmatica*) was in contact with diaphragm and right abdominal wall. This surface was covered by the peritoneum (*peritoneum*). The falciform ligament (*lig. falciforme*) was a thin peritoneal fold and it was attached to the convex surface of the diaphragm and the caudal surface of the right abdominal wall. The visceral (Figure 4), concave surface (*facies visceralis*) was in relation to the guts (stomach, duodenum, right colic flexure, the pancreas, the right kidney, and suprarenal gland).

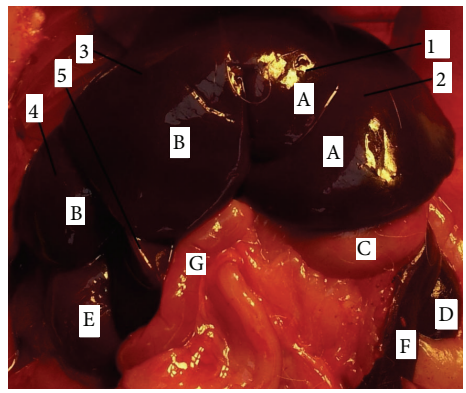

Figure 3: Morphology of rat liver (*facies diaphragmatica*). 1: *lobus hepatis sinister medialis*, 2: *lobus hepatis sinister lateralis*, 3: *lobus hepatis dexter medialis*, 4: *lobus hepatis dexter lateralis*, 5: *lobus quadratus*, A: *lobus hepatis sinister*, B: *lobus hepatis dexter*, C: *ventriculus*, D: *ren dexter*, E: *ren sinister*, F: *lien*, and G: *intestinum*.

The diaphragmatic and visceral surfaces were fused in four margins. On dorsal margin (*margo dorsalis*) of the liver was situated caudal vena cava (*v. cava caudalis*) in groove for this vein (*sulcus venae cavae*). This vein received segmental hepatic veins (*vv. hepaticae*) from the liver tissue. Dorsally to the caudal vena cava coronary ligament was situated (*lig.* coronarium) which was divided into two branches. The right branch continued into the right triangular ligament (*ligamentum triangulare dextrum*) and the left branch continued into the left triangular ligament (*ligamentum triangulare sinistrum*). On the ventral margin (*margo ventralis*) was hepatic teres ligament of the liver (*ligamentum teres hepatis*) was embedded into deep fissure, which was presented between left medial lobe and quadrate lobe. Ligament of the liver (*ligamentum teres hepatis*) formed the main continuation of the falciform ligament and it presented the obliterated rest of umbilical vein (*v. umbilicalis*). Between the liver and

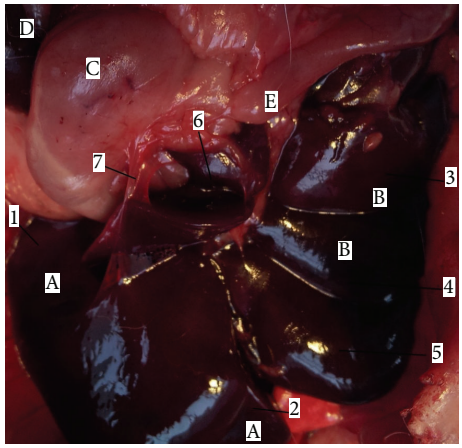

Figure 4: Morphology of rat liver (*facies visceralis*). 1: *lobus hepatis sinister lateralis*, 2: *lobus hepatis sinister medialis*, 3: *lobus hepatis dexter lateralis*, 4: *lobus hepatis dexter medialis*, 5: *lobus quadratus*, 6: *lobus caudatus*, 7: *lig. hepatogastricum*, A: *lobus hepatis sinister*, B: *lobus hepatis dexter*, C: *ventriculus*, D: *lien*, and E: *intestinum*.

other organs (duodenum, stomach) were ligaments, which formed the lesser omentum (*omentum minus*). There were hepatoduodenal and hepatogastric ligaments (*ligamentum hepatoduodenale* and *hepatogastricum*). On the right and left side of the liver were the right and left margins (*margo dexter* et sinister), which bear deep fissures between the lobes. On the visceral surface was the transverse fissure of the liver (*porta hepatis*). The transverse fissure of the liver (*porta hepatis*) was the opening where entered portal vein, hepatic artery and nerves and came out hepatic duct. The laboratory rats had these six lobes of the liver: left medial, lateral and right medial, lateral lobe, caudate and quadrate lobe (*lobus hepatis sinister medialis*, *lateralis* and *lobus hepatis dexter medialis*, *lateralis*, *lobus caudatus*, and *quadratus*). Between hepatic lobes were presented deep fissures. The caudate lobe was divided into caudate and papillar process (*processus caudatus* and *papillaris*). The pointed caudate process arose from the visceral surface and it protruded dorsally, to the right side of the liver. The caudate process met the right kidney. Papillary process (*processus papillaris*) extended across the *curvatura ventriculi major* and it was in contact with the *facies visceralis* of the stomach. The gall bladder (*vesica fellea*) was absent. All hepatic ducts were fused and formed common hepatic duct (*ductus hepaticus communis*), which led to the duodenum. The common hepatic duct was situated ventrally and to the right of the portal vein (*vena portae*).

3.3. Intestine Anatomy. The small intestine (*intestinum tenue*) of the laboratory rat arose from pyloric part of the stomach (*pars pylorica ventriculi*). The pyloric part was situated in the right side to the median plane. The first part of the small intestine, duodenum (*duodenum*), arose from the stomach. The length of duodenum was approximately 95–100 mm (Table 3). Cranial part of duodenum (*pars cranialis duodeni*) is a part, which was situated near the visceral surface of the liver and right abdominal wall. Descending part of duodenum (*pars descendens duodeni*) continued by the right abdominal wall to the right kidney. Between cranial duodenal part and descending part of duodenum was the first flexure, cranial duodenal flexure (*flexura duodeni cranialis*). The descending part of the duodenum turned to the cranial direction, as an ascending part of duodenum (*pars ascendens duodeni*) in this position. Between the descending and ascending part of duodenum was presented the caudal duodenal flexure (*flexura duodeni caudalis*). The ascending part of the duodenum continued to the median plane to the jejunum (*jejunum*). The other flexure was between the last part of duodenum and jejunum and it was duodenojejunal flexure (*flexura duodenojejunalis*). This part of small intestine filled the right part of the abdominal cavity. *Jejunum* formed loops and it filled the right part of the abdominal cavity ventrally and it passed fluently to the ileum (*ileum*). The jejunum measured 890–1300 mm (Table 3). At the level, where ileum entered to the cecum, enlargement was presented, *sacculus rotundus*. *Sacculus rotundus* was formed by lymphatic tissue. The length of the ileum was 20–30 mm (Table 3). The opening of the ileum into the cecum was enclosed to the beginning of the colon (*colon*). The cecum was on the right caudal side of the abdominal cavity. The cecum had base (*basis*), body (*corpus*), and an apical part (*apex*). From the apex continued *processus vermiformis*, which was the last part of the *cecum*. The cecum had the long mesentery which allowed for different positional variation. Behind the *cecum*, which measured 45–65 mm (Table 3), followed the *colon*. The first part was ascending colon (*colon ascendens*); it led cranially to the thoracic cavity. From the left to the right side ran tansverse colon (*colon transversum*), the second part of the colon. On the right side of the abdominal cavity continued descending colon (*colon descendens*) (Figure 5). The length of the colon was 95–100 mm (Table 3) and rectum measured approximately 75 mm (Table 3). The rectum ran as a straight tube through the pelvis and ended just below the root of the anus. Behind rectum were anal canal (*canalis analis*) and anus (*anus*).

3.4. The Division of the A. Coeliaca. The results of our study indicated that the stomach, liver, and intestine in a laboratory rat are supplied by the three main arteries, which are the branches of the abdominal aorta. These arteries are celiac artery (*a. coeliaca*), cranial mesenteric artery (*a. mesenterica cranialis*), and caudal mesenteric artery (*a. mesenterica caudalis*).

Arteria coeliaca was the first visceral branch, which left the ventral wall of the abdominal aorta (Figure 6). It was the short, unpaired trunk, which arose at the level of the third lumbar vertebra. This artery supplied stomach, spleen, liver, pancreas, and cranial part of the duodenum. The coeliac artery was divided into three main branches: splenic artery (*a. lienalis*), left gastric artery (*a. gastrica sinistra*), and hepatic artery (*a. hepatica*).

Splenic artery (*arteria lienalis*) continued after the origin on the left side, by the cranial border of the left pancreatic lobe. There was *arteria lienalis* devided into many pancreatic branches (*rr. pancreatici*) and than continued ventral to the centre of the splenic hilus. The splenic artery gave the common trunk for the splenic branches (*rr. lienales*) of

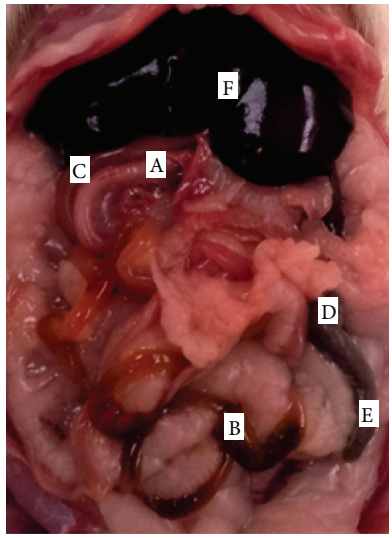

Figure 5: Abdominal cavity with intestine in rat, ventral view. A. *duodenum*, B. *jejunum*, C. *colon*, D. *cecum*, E. *processus vermiformis*, and F. *hepar*.

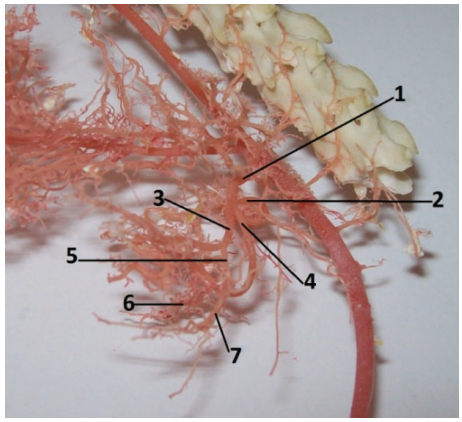

Figure 6: The division of the coeliac artery in rat. 1: *a. celiaca*, 2: *a. gastrica sinistra*, 3: *a. hepatica*, 4: *a. lienalis*, 5: *a. gastroduodenalis*, 6: *a. gastroepiploica dextra*, and 7: *a. pancreaticoduodenalis cranialis* (casting medium Duracryl Dental).

the spleen. Our results indicated that *rr. lienales* were presented in numbers 5, 6, and 8 (5 in 20%, 6 in 30%, and 8 in 50%). Splenic artery then continued as the left gastroepiploic artery (*a. gastroepiploica sinistra*) on the greater curvature of the stomach. This left gastroepiploic artery was divided into short gastric arteries (*aa. gastricae breves*), which supplied the fundic region of the greater curvature of the stomach. These short gastric arteries were visible on gastric surfaces.

Left gastric artery (*arteria gastrica sinistra*) originated directly from the coeliac artery in all cases. The left gastric artery directed on the lesser curvature of the stomach in the region of the insertion of the gastric mesentery (*mesogastrium*). Along this course it gave off to the parietal branches (*rr. parietales*) and *viscerales* to the surfaces of the stomach. These short gastric branches anastomosed each other.

The last branch of the celiac artery was *a. hepatica*. It turned to the liver and gave rise to the hepatic branches, the *rr. pancreatici*, the right gastric artery (*a. gastrica dextra*), and the gastroduodenal artery (*a. gastroduodenalis*). The last branch was the parent artery of the cranial pancreaticoduodenal artery (*a. pancreaticoduodenalis cranialis*) and the right gastroepiploic artery (*a. gastroepiploica dextra*). Right gastric artery (*a. gastrica dextra*) directed on the lesser curvature of the stomach; it supplied the pyloric region. This artery followed the lesser curvature of stomach (*curvatura ventriculi minor*) and there it anastomosed with the left gastric artery. Right gastric artery gave off parietal and visceral branches to both gastric surfaces. Gastroduodenal artery (*a. gastroduodenalis*) was the common part for the greater curvature of the stomach and for the first part of duodenum. Right gastroepiploic artery was the terminal limb of the gastroduodenal artery, which was divided into the short gastric arteries for parietal and visceral surfaces of the stomach. Right gastroepiploic artery and *sinistra* anastomosed each other. Cranial pancreaticoduodenal artery was one of the branches of the gastroduodenal artery. This artery directed in the mesoduodenum (*mesoduodenum*) along the cranial and descending parts of the duodenum.

3.5. The Division of the Cranial Mesenteric Artery. Our results indicated that cranial mesenteric artery (*a. mesenterica cranialis*) was the thickest branch of the abdominal aorta. It was the unpaired second visceral branch from the ventral wall of the abdominal aorta, caudal to the celiac artery. This artery is more important during embryonic development, because the gut twists around this artery. The cranial mesenteric artery supplied all parts of digestive system which were attached to this mesentery and which participate in the embryonic rotation. The following variations were observed in the origin of the cranial mesenteric artery. In 9% of the corrosion cast specimens, the cranial mesenteric artery (*a. mesenterica cranialis*) originated from the abdominal aorta cranial to the origin of the right renal artery (*a. renalis dextra*) (Figure 10), and in 37% a common trunk originated from the abdominal aorta for the cranial mesenteric artery and the right renal artery (*a. renalis dextra*) (Figure 9). In 39% the *a. mesenterica cranialis* originated from abdominal aorta (*aorta abdominalis*) caudal to the right renal artery (Figure 8). Cranial mesenteric artery was divided into these branches: middle colic artery (*a. colica media*), caudal pancreaticoduodenal artery (*a. pancreaticoduodenalis caudalis*), right colic artery (*a. colica dextra*), jejunal arteries (*aa. jejunales*), and ileocecocolic artery (*a. ileocecocolica*).

The results of our study indicated that *a. colica media* was the first branch, running caudal from its origin. It supplied blood to the transverse colon and cranial portion of the descending colon. In 46% it branched off individually from the cranial mesenteric artery and in 37% as a common trunk with the right colic artery.

Inferior pancreaticoduodenal artery (*arteria pancreaticoduodenalis caudalis*) left the cranial mesentery artery in caudal direction and coursed in the mesentery of the ascending part of duodenum to the caudal duodenal flexure. This artery originated ventral to the right colic artery in laboratory

Table 1: Morphological comparison of the rat and human stomach.

Rat stomach	Human stomach
Semilunar shaped sac	Pear-shaped sac
Fundus ventriculi forms blind ventricular sac	Only simple *fundus ventriculi*
The mucous membrane is divided into glandular part and nonglandular part	The mucous membrane is simple and consists of glandular part
Weight 1,8% of the total body weight	Weight 6,2% of the total body weight
Long celiac trunk	Short celiac trunk

rat. Inferior pancreaticoduodenal artery was divided into pancreatic branches (*rr. pancreatici*) for pancreas and duodenal branches (*rr. duodenales*) for *duodenum*. The following variations were observed in the origin of this artery. In 46% the caudal pancreaticoduodenal artery was originated cranially from the right colic artery and in 37% after the right colic artery. On the corrosion cast specimens, we noticed the presence of the anastomosis between inferior pancreaticoduodenal artery and artery superior pancreaticoduodenal (*a. pancreaticoduodenalis cranialis*).

The third branch of the cranial mesenteric artery in laboratory rat was the right colic artery. This artery ran towards the caudal part of the ascending colon. In 37% right colic artery originated from the cranial mesenteric artery as a common trunk with the middle colic artery before the origin of the inferior pancreaticoduodenal artery. In 46% right colic artery branched off independently after the origin of inferior pancreaticoduodenal artery.

Jejunal arteries originated from the cranial mesenteric artery in higher number. These arteries supplied blood to the jejunum and cranial part of ileum. Jejunal arteries ran in the mesojejunum (*mesojejunum*). On the corrosion cast specimens, we noticed the presence of a jejunal arcade and a jejunal trunk. Jejunal trunks were the common parts for the origin of the jejunal arteries from the cranial mesentery artery. Jejunal arcades were presented on the intestinal wall and they were the terminal parts of the jejunal arteries.

Arteria ileocecocolica arose from the cranial mesenteric artery; it directed caudoventrally; it coursed to the cecocolic junction. The principal continuation of the cranial mesenteric artery in laboratory rat was the *a. ileocecocolica*, which was divided into the colic branch (*r. colicus*) for ascending colon, ileal artery (*a. ilealis*) for ileum, and cecal artery (*a. cecalis*) for *cecum*. The presence of the *a. ileocecocolica* together with its branches was observed in 46%. Cranial mesenteric artery in 37% continued as the ileocecal artery (*a. ileocecalis*), which is bifurcated into the cecal artery (*a. cecalis*) and ileal artery and colic branch branched off independently.

3.6. The Division of the Caudal Mesenteric Artery. Caudal mesenteric artery (*arteria mesenterica caudalis*) was the third unpaired visceral branch to spring from the ventral wall of the abdominal aorta. This artery was a thinner branch than the cranial mesenteric artery. Caudal mesenteric artery was divided into left colic artery (*a. colica sinistra*) and cranial rectal artery (*a. rectalis cranialis*). This artery supplied blood to the caudal part of the descending colon and the rectum (Figure 11).

Our results described anastomosis between cranial pancreaticoduodenal artery and caudal pancreaticoduodenal artery. We investigated the second anastomosis between middle colic artery and left colic artery (Figure 12).

Table 2: Morphological comparison of the rat and human liver.

Rat liver	Human liver
Six lobes of the liver	No clear morphological division of the lobes
Dividing of caudate lobe is obvious	No clear subdivision of the caudate lobe
Weight 5% of the total body weight	Weight 2,5% of the total body weight
Gall bladder is absent	Gall bladder is present

Table 3: Length and diameters of parts of rat intestinal tract [12].

	Length (mm)	Diameter (mm)
Duodenum	95–100	2,5–3
Jejunum	900–1350	4-5
Ileum	25–35	3–5
Cecum	50–70	10
Colon	90–110	10–3
Rectum	80	3–10

4. Discussion

The detailed description of the rat organs of the digestive system and their vasculature is decisive for ischemia and transplantations in this model of laboratory animals. Many previous publications and descriptions have mentioned only partial results of anatomy of these organs. We have presented integrated knowledge about a rat stomach, liver, intestine, and vasculature, which is more important for experimental ischemia and transplantations. Many authors described microstructures of these organs [13, 14]. The anatomical relationship between the human and rat digestive organs is still undefined (Tables 1, 2, and 4). The functional anatomy of the digestive organs in the rat is considered similar to humans. The rats are suitable for determining the mechanism of drug absorption and bioavailability values from powder or solution formulations [14].

Table 4: Morphological comparison of the rat and human intestine.

Rat intestine	Human intestine
Length 1745 mm	Length 6-7 m
Small intestine 1485 mm	Small intestine 5,5–6,4 m
Large intestine 260 mm	Large intestine 1,5 m

The classic morphological books describe the human stomach as the most dilated part of the digestive system, beneath the diaphragm in the left hypochondriac and epigastric region of the abdominal cavity [15, 16]. Its shape and topography are closely associated with organogenesis. Some developmental abnormality of the stomach, as well as abnormality of vessels and nerves, may influence stomach morphology [17, 18]. The empty stomach has a typical, cylindrical shape with a well-formed anterior and posterior wall and lesser and greater curvature as well as fundus, cardia, body, and pylorus [19]. In distended one, the anterior wall increases the area attached to the abdominal wall. During inspiration stomach is displaced downward. Abnormal fluid accumulation in the pleural or peritoneal cavity may change the stomach shape as well [16].

The general description of the human stomach [20] indicates that the cardiac part of the stomach is situated on the left side at the level of the tenth thoracic vertebra. Near the cardiac part is *fundus ventriculi*, which is simple and is filled by air. At the opposite end of the stomach is pyloric part, which has a very strong band of the smooth muscle, pyloric sphincter. In the human there is only the glandular type of the stomach and it is lined with cardiac, gastric, and pyloric mucosa (Figure 2). From domestic mammals only carnivores have the same gastric mucous membrane as a human [21]. Some species do not possess a glandular stomach and in that case the oesophageal mucosa becomes continuous with that of the small intestine [22]. Stomach is supplied by vessels of the short celiac trunk (Figure 7). The lesser curvature supplied primarily the left gastric artery which arose from celiac trunk. The right gastric artery was usually a small vessel that provided branches to the first part of duodenum and the pylorus. The right and left gastroepiploic arteries arose from the gastroduodenal and splenic arteries, respectively. They from an arcade along the greater curvature, the right provided blood to the antrum, and the left supplied the lower portion of the fundus. The short gastric arteries that arose from the splenic artery were small and relatively insignificant in terms of the amount of blood that they delivered to the most proximal portion of the body of the stomach [23]. The anatomy of the rat stomach is greatly influenced by adaptation, nature of food, body size, and shape. The division and description of the rat stomach are not uniform. The rat stomach is divided into the forestomach (pars proventricularis) and glandular stomach (corpus or pars glandularis) (Figure 2). The forestomach occupies about three-fifths of the stomach area. The glandular stomach is the other part and is divided into the fundus and pylorus [24]. The morphology of the rodent stomach is different from the stomach of other laboratory animals [25, 26]. Functionally, the forestomach serves as a storage organ [27]. The glandular

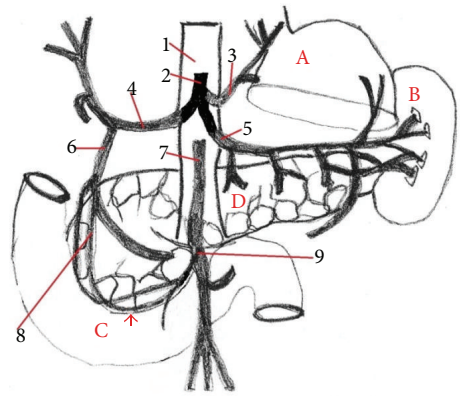

Figure 7: The division of the celiac trunk and the presence of the anastomosis between *a. supraduodenalis superior* and *a. pancreaticoduodenalis inferior* in human (anastomosis, *arrow*) (scheme). 1: *aorta abdominalis*, 2: *truncus ceoliacus*, 3: *a. gastrica sinistra*, 4: *a. hepatica communis*, 5: *a. splenica*, 6: *a. gastroduodenalis*, 7: *a. mesenterica superior*, 8: *a. supraduodenalis superior*, 9: *a. pancreaticoduodenalis inferior*, A: liver, B: spleen, C: duodenum, and D: pancreas.

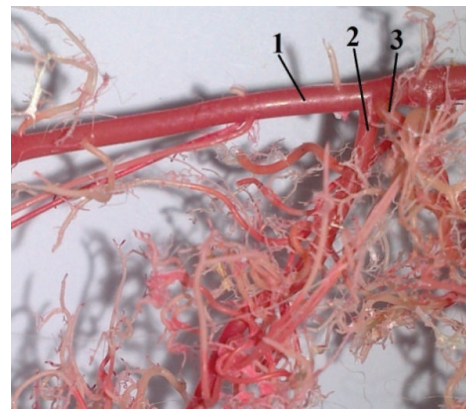

Figure 8: Photograph demonstrating the origin of *a. mesenterica cranialis* caudal to the *a. renalis dextra*. 1: *aorta abdominalis*, 2: *a. mesenterica cranialis*, and 3: *a. renalis dextra* (casting medium Duracryl Dental).

stomach is functionally similar to that of the other laboratory animals [28]. The nomenclature is not the same. Some authors describe the rat stomach glandular and nonglandular parts, which are separated by the limiting ridge [29]. The rat stomach is supplied by long celiac trunk [30, 31]. The origin, division, and course of the celiac trunk (Figure 6) and its branches are similar to human, but the area of supplying of single arteries is different. The experiment of the blood supply to the gastric mucosa in rats showed these results. The left gastric artery was manifest at two sites, the greater curvature, the pylorus, and the first part of duodenum. The right gastric artery was very small and terminated by supplying the duodenum. The second largest artery after the left gastric artery was the right gastroepiploic artery which supplied most of the greater curvature. The fundus received a few small branches from the splenic artery [32].

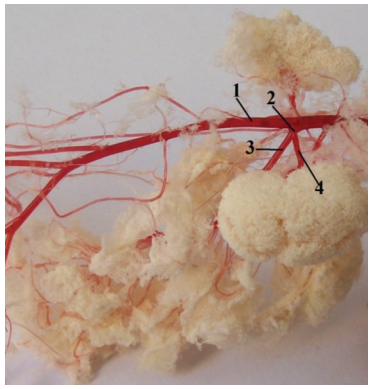

Figure 9: Photograph showing the common trunk of the *a. mesenterica cranialis* with the *a. renalis dextra*. 1: *aorta abdominalis*, 2: a common trunk, 3: *a. mesenterica cranialis*, and 4: *a. renalis dextra* (casting medium PUR SP).

Figure 10: Photograph pointing out the origin of the *a. mesenterica cranial* is cranial to the *a. renalis dextra*. 1: *aorta abdominalis*, 2: *a. mesenterica cranialis*, 3: *a. renalis dextra*, and 4: *a. testicularis dextra* (casting medium Duracryl Dental).

The morphology of the rat liver has been considered different from humans [33]. The general description of the liver in human segmentation by Couinaud divided the human liver into eight lobes and this presents the basis of hepatic resections [34]. Three hepatic veins divide the liver into four parts (right lateral part, right paramedian part, left paramedian part, and left lateral part). Each part receives a portal branch which bifurcates and drains the blood from each lobe [35]. The nomenclature of the liver in rats is not identical [36–38]. Most authors divided the rat liver into these four lobes: left lateral lobe, medial lobe (left and right medial), caudate lobe (anterior or inferior, posterior, or superior), and right lobe. Popesko et al. divided the rat liver into six lobes: right hepatic lobe (lateral and medial part), left hepatic lobe (lateral and medial lobe), caudate lobe (caudate and papillary process), and quadrate lobe [39] (Figures 3 and 4).

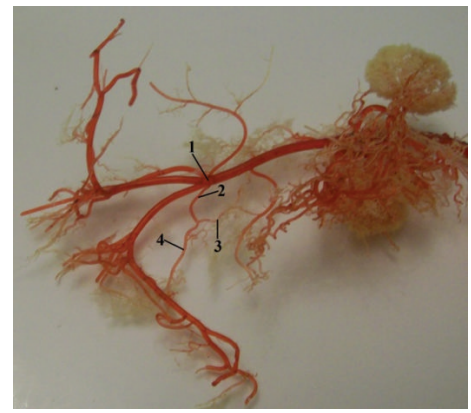

Figure 11: Photograph focusing the division of the *a. mesenterica caudalis*. 1: *aorta abdominalis*, 2: *a. mesenterica caudalis*, 3: *a. colica sinistra*, and 4: *a. rectalis cranialis* (casting medium PUR SP).

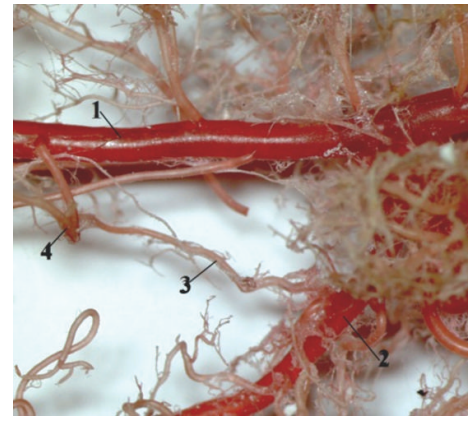

Figure 12: The presence of the anastomosis between *a. colica sinistra* and *a. colica media*. 1: *aorta abdominalis*, 2: *a. mesenterica cranialis*, 3: *a. colica media*, and 4: *a. colica sinistra* (casting medium Duracryl Dental).

Lorente et al. divided the rat liver into two parts: superior and inferior liver and six sectors: caudate process, caudate lobe, right lateral lobe, right segment of the right medial lobe, middle, and left segment of the right medial lobe and left medial lobe; the last sector is the left lateral lobe [33]. In the study of Kogure et al., it was shown that the liver of the rat is the same as that of the humans [36]. Each lobe of the rat liver has its own arteries. These unrivalled considerations of the rat liver morphology make resections of different extents simply and highly reproducible and of low price when performing experiments [40]. Different from humans, other mammals, the rat liver does not have a gallbladder, though some mammals, like the horse, deer, or some birds, like the pigeon, do not have it either [41].

The functional anatomy of the intestine in the rat has been considered similar to humans. The length of the human intestine and its parts is not uniform.

The small intestine of human is from the morphophysiological aspect the main organ of digestion in the body. This part of intestine is divided into duodenum, jejunum,

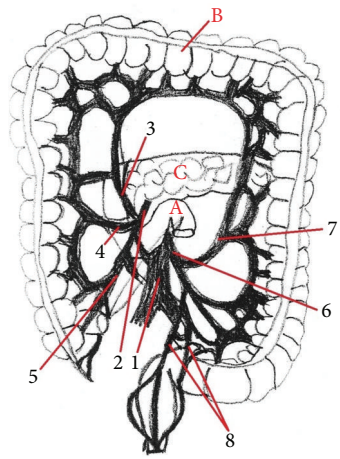

Figure 13: The division of the a. *mesenterica superior* in human. 1: *aorta abdominalis*, 2: *a. mesenterica inferior*, 3: *a. colica media*, 4: *a. colica dextra*, 5: *a. ileocolica*, 6: *a. mesenterica inferior*, 7: *a. colica sinistra*, 8: *aa. rectales superiores*, A: *flexura duodenojejunalis*, B: *colon*, and C: *pancreas*.

and ileum. The length of the small intestine in a healthy human is approximately 5,5–6,4 m (Table 4). The minimum length of the intestine, which is needed for maintenance of the absorption, is 50–70 cm, while the full function of the large intestine is carried on. Diseases of small intestine and their vasculature form acute or chronic ischemia of intestine. The effect of these disorders is in many cases the resection of the intestinal part. It is mainly the part of jejunum and *ileum*. If you take off the intestine, there will be problems with malabsorption and maldigestion and other failures of digestion of the base of the nutriments. If these cases are not treated, the disease can eventually cause multiple organ failure [42]. The anatomical nomenclature of the intestinal supply in humans and rats is different. The rat small intestine is supplied by cranial mesenteric artery [30, 31] and by superior mesenteric artery in human [43]. The main division of this artery is the same. Some authors described in humans the anastomosis between a. ileocolica and a. colica dextra. In 5% of cases this anastomosis was absent [44]. The second anastomosis was between *a. supraduodenalis superior* and *a. pancreaticoduodenalis inferior* (Figure 13). In rats anastomosis was described between cranial pancreaticoduodenal artery and caudal pancreaticoduodenal artery (Figure 12). Our results of this anastomosis agreed with other authors [30, 45].

The large intestine is the part which is more important for absorption of water, electrolytes, and vitamins. Humans have a poorly defined cecum, which is only continuous with the colon [14]. But the position of the appendix of the cecum is very different in human. The cecum of pig is more complex than humans. In pig cecum, we can describe three longitudinal bands and three orders of sacs or haustra [21]. The rat cecum is as large as rat stomach.

The human colon played a major role in the absorption of water, salt (Na^+, Cl^-), and other minerals. This part of human intestine consists of the ascending, transverse, descending, and sigmoid sections. All parts of colon in human are sacculated. The sacculation of the colon in human is similar to pigs [14]. The rat colon is not sacculated, and it is so simple. Our results from the rat colon are in agreement with other authors [12, 31, 39]. The supplying of the large intestine in human and in rats is the same. Only the nomenclature of this artery is different. We called this artery *arteria mesenterica inferior* in human [43] and caudal mesenteric artery in rats [30, 31]. Some authors described anastomosis between left colic artery and middle colic artery. This anastomosis was present in 32%, and it was absent in 7%. More authors described in research the presence of meandering mesenteric artery, arc of Riolan [46]. This artery was an additional pathway between the superior and inferior mesenteric artery in human [47].

The blood supplying of the gastrointestinal tract is more important for surgical treatment in acute or chronic mesenteric ischemia or organ transplantation in human.

5. Conclusions

In summary, the rat is the most extensively used experimental animal model in veterinary and human surgical research of the abdominal cavity, intestinal transplantation, or the study of medicaments absorption. The progress of the surgical therapeutic methods (treatment of ischemic injury of stomach, liver, intestine, surgical resection, the congenital disorders, Crohn's disease, transplantation) and the study of stomach, liver, and intestine morphology, function, and diseases depend on new knowledge of these organs and their vasculature. We investigated the functional anatomy of the stomach, liver, and intestine and their vasculature of the laboratory rat and compared it with the human morphology. Compared to humans, the laboratory rats have similar anatomical structure of the stomach, liver, and intestine; therefore, nowadays, the laboratory rat is one of the most popular models for research of anatomical, physiological, and biochemical relations in the digestive system. Nevertheless, the relationship (function, anatomy) between them is not defined.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This study was supported by the project of the Slovak Ministry of Education, Grant no. VEGA 1/0111/13.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 274534, 6 pages
http://dx.doi.org/10.1155/2015/274534

Research Article

Serum Liver Fibrosis Markers for Predicting the Presence of Gastroesophageal Varices in Liver Cirrhosis: A Retrospective Cross-Sectional Study

Xingshun Qi, Hongyu Li, Jiang Chen, Chunlian Xia, Ying Peng, Junna Dai, Yue Hou, Han Deng, Jing Li, and Xiaozhong Guo

Liver Cirrhosis Study Group, Department of Gastroenterology, General Hospital of Shenyang Military Area, 83 Wenhua Road, Shenyang 110840, China

Correspondence should be addressed to Xiaozhong Guo; guo_xiao_zhong@126.com

Received 23 January 2015; Revised 23 April 2015; Accepted 28 April 2015

Academic Editor: Marcela Kopacova

Background and Aims. A retrospective cross-sectional study was conducted to evaluate the role of hyaluronic acid (HA), laminin (LN), amino-terminal propeptide of type III procollagen (PIIINP), and collagen IV (CIV) in predicting the presence of gastroesophageal varices (GEVs) in patients with liver cirrhosis. *Methods*. We enrolled 118 patients with liver cirrhosis who underwent the tests for the four serum liver fibrosis markers and upper gastrointestinal endoscopy at the same admissions. The predictive values of the four serum liver fibrosis markers were evaluated by the areas under the receiving operator characteristics curves (AUROCs) with 95% confidence intervals (CIs). *Results*. The prevalence of GEVs was 88% (104/118). The AUROCs for HA, LN, PIIINP, and CIV levels in predicting the presence of GEVs were 0.553 (95% CI: 0.458 to 0.644, $P = 0.5668$), 0.490 (95% CI: 0.397 to 0.584, $P = 0.9065$), 0.622 (95% CI: 0.528 to 0.710, $P = 0.1099$), and 0.560 (95% CI: 0.466 to 0.652, $P = 0.4909$). The PIIINP level at a cut-off value of 31.25 had a sensitivity of 73.1% and a specificity of 57.1%. *Conclusions*. The present study did not recommend HA, LN, PIIINP, and CIV levels to evaluate the presence of GEVs in liver cirrhosis.

1. Introduction

Variceal bleeding is the most common life-threatening complication of liver cirrhosis [1]. Routine follow-up surveillance for gastroesophageal varices (GEVs) in such patients is important for establishing an early diagnosis and adopting the preventive measures. Upper gastrointestinal endoscopy is the golden diagnostic method for GEVs in our everyday clinical practice. According to the current consensus and practice guidelines recommendations, once liver cirrhosis is diagnosed, the patients should undergo the screening endoscopy for assessing GEVs [2, 3]. If the varices are lacking, the patients should undergo the endoscopy every 2-3 years, and if the varices are at a low risk, they should undergo the endoscopy every 1-2 years. But the endoscopy has its limitations, such as invasiveness, relatively high cost, procedure-related complications, poor patients' compliance, and high demand of endoscopists' skills. The limitations become more obvious in patients who need repeated endoscopy. Nowadays, noninvasive diagnostic tests of GEVs have been widely explored [4, 5]. They primarily include the liver and spleen stiffness measurement [6–10], platelet count/spleen diameter ratio [11, 12], aspartate aminotransferase/platelets count ratio [13, 14], portal venous haemodynamic parameters [15], and indocyanine green "15-minute" retention test, or other liver function tests [16]. Indeed, these noninvasive markers for the diagnosis of GEVs are also frequently used to reflect the severity of liver fibrosis [17, 18]. Thus, it appears to be reasonable to assume that hyaluronic acid (HA), laminin (LN), amino-terminal propeptide of type III procollagen (PIIINP), and collagen IV (CIV), which are four major serum markers for liver fibrosis, are also eligible for the assessment of GEVs.

Until now, few studies have evaluated the role of serum liver fibrosis markers in predicting the presence of GEVs in patients with liver cirrhosis. Gressner et al. evaluated the

correlation of LN and PIIINP concentrations with portal venous pressure in patients with liver fibrosis ($n = 21$) and cirrhosis ($n = 12$) [19]. They found a positive correlation between LN and portal venous pressure. The same study team further confirmed the diagnostic accuracy of HA and LN in the assessment of portal hypertension [20]. Kondo et al. also found a positive and significant correlation between LN and hepatic vein pressure gradient in 20 patients with alcoholic liver cirrhosis [21]. However, these investigators suggested that LN should not be a reliable marker for assessing the risk of variceal bleeding. Generally, the limited evidence originates from old studies with small sample sizes and heterogeneous study population. Herein, we conducted a retrospective cross-sectional study to evaluate the predictive values of serum liver fibrosis markers (i.e., HA, LN, PIIINP, and CIV) for the presence of GEVs.

2. Methods

2.1. Study Design. In this study, the eligibility criteria were as follows: (1) patients were admitted to the General Hospital of Shenyang Military Area between January 2013 and June 2014; (2) patients were diagnosed with liver cirrhosis; (3) patients underwent the tests for the four serum liver fibrosis markers (i.e., HA, LN, PIIINP, and CIV) at their admissions; and (4) patients underwent upper gastrointestinal endoscopy to evaluate the presence of varices at the same admissions. This study was conceived by two investigators (Qi X. and Guo X.), and the study protocol was approved by the Medical Ethical Committee of the General Hospital of Shenyang Military Area (number k(2015)03).

Electronic medical charts were retrospectively reviewed. The following data were collected: age, sex, presence of malignancy, etiology of liver cirrhosis, clinical presentation, red blood cell (RBC), hemoglobin (Hb), white blood cell (WBC), platelets count (PLT), total bilirubin (TBIL), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamine transferase (GGT), blood urea nitrogen (BUN), creatinine (Cr), prothrombin time (PT), activated partial thromboplastin time (APTT), international normalized ratio (INR), severity of liver dysfunction (Child-Pugh and model for the end-stage of liver diseases score), and HA, LN, PIIINP, and CIV levels. The laboratory tests for the HA, LN, PIIINP, and CIV were performed by using the chemiluminescent immunoassay in the LUmo Microplate Luminometer equipment. The diagnostic kits for the HA, LN, PIIINP, and CIV were also purchased from the Autobio Diagnostics Co., Ltd. (Zhengzhou, Henan Province, China). The reference values were as follows: HA < 120 ng/mL, LN < 130 ng/mL, PIIINP < 15 ng/mL, and CIV < 95 ng/mL. Other laboratory tests were performed in the Department of Clinical Laboratory of our hospital.

Severity of EVs was classified into no, mild, and moderate/severe according to the 2008 Hangzhou consensus. It was jointly published in Chinese language by the Chinese Society of Gastroenterology, Chinese Society of Hepatology, and Chinese Society of Digestive Endoscopy. Briefly, if there were red color signs, the varices were considered as moderate/severe. Otherwise, the forms of varices were further identified. If the forms were linear, they were considered as mild. If the forms were snake-like, they were considered as moderate. If the forms were bead-like or tubercular, they were considered as severe.

2.2. Statistical Analyses. Categorical and continuous variables were reported as the frequencies and means ± standard errors, respectively. Continuous variables were compared by the independent sample t-tests. Receiving operator characteristics (ROC) curve analysis was performed to identify the discriminative capacity of HA, LN, PIIINP, and CIV levels in predicting the presence of GEVs. A cut-off value of HA, LN, PIIINP, or CIV level was chosen as both sensitivity and specificity were optimal. Areas under the ROC curves (AUROCs) with 95% confidence intervals (CIs) were also reported. $P < 0.05$ was considered statistically significant. All statistical analyses were performed by using the MedCalc software version 11.4.2.0 and SPSS Statistics version 17.0.0.

3. Results

During this period, a total of 272 patients with liver cirrhosis underwent the tests for the HA, LN, PIIINP, and CIV levels. Notably, in our previous study, 228 of them had been enrolled to evaluate the correlation of the four serum liver fibrosis markers with the severity of liver dysfunction [22]. In the present study, 118 of them were enrolled to evaluate the correlation of serum liver fibrosis markers with the presence of GEVs (Table 1), because 154 patients did not undergo upper gastrointestinal endoscopy at admissions. Among these included patients, 10 had a diagnosis of hepatocellular carcinoma, and 3 had previous history of other malignancy (thyroid carcinoma $n = 1$, renal carcinoma $n = 1$, and rectal cancer $n = 1$). The major causes of hospitalization included upper gastrointestinal bleeding ($n = 69$), weakness ($n = 18$), massive ascites ($n = 10$), abdominal distension ($n = 6$), abdominal pain ($n = 6$), jaundice ($n = 4$), incident diagnosis of liver cirrhosis ($n = 3$), hepatic encephalopathy ($n = 1$), and gingival bleeding ($n = 1$). The etiology of liver cirrhosis included hepatitis B virus infection alone ($n = 41$), hepatitis C virus infection alone ($n = 11$), a combination of hepatitis B virus and hepatitis C virus infections ($n = 1$), alcohol alone ($n = 27$), a combination of hepatitis B virus and alcohol ($n = 4$), autoimmune hepatitis ($n = 3$), drug-related ($n = 5$), and unknown ($n = 26$). The prevalence of GEVs was 88% (104/118). GEVs were severe, moderate, and mild in 46, 21, and 16 patients, respectively. Notably, the severity of GEVs was unclear in 21 patients. The location of GEVs included esophageal varices alone ($n = 48$), esophageal and gastric varices ($n = 52$), and gastric varices alone ($n = 4$).

HA level was not significantly different between patients with and without GEVs (621.21 ± 117.21 versus 1511.77 ± 1137.21). The AUROC for HA level in predicting the presence of GEVs was 0.553 ± 0.0917 (95% CI: 0.458 to 0.644, $P = 0.5668$) (Figure 1). The best cut-off value was 299.24 with a sensitivity of 60.6% and a specificity of 57.1%.

LN level was not significantly different between patients with and without GEVs (142.86±10.31 versus 133.60±23.84). The AUROC for LN level in predicting the presence of GEVs

Table 1: Characteristics of patients.

Variables	Values
Age (years)	55.14 ± 0.98
Sex (male/female): *n*	76/42
Hepatocellular carcinoma (yes/no): *n*	10/108
Ascites (yes/no); *n*.	52/66
Hepatic encephalopathy (yes/no): *n*	2/116
Gastroesophageal varices (yes/no): *n*	104/14
Red blood cell (10^{12}/L)	3.15 ± 0.07
Hemoglobin (g/L)	89.68 ± 2.52
White blood cell (10^9/L)	4.16 ± 0.24
Platelets count (10^9/L)	91.11 ± 5.71
Total bilirubin (umol/L)	26.90 ± 2.37
Albumin (g/L)	32.92 ± 0.58
Alanine aminotransferase (U/L)	46.91 ± 10.91
Aspartate aminotransferase (U/L)	60.64 ± 7.43
Alkaline phosphatase (U/L)	108.12 ± 6.93
Gamma-glutamine transferase (U/L)	134.29 ± 40.40
Blood urea nitrogen (mmol/L)	6.01 ± 0.32
Creatinine (umol/L)	63.72 ± 3.06
Prothrombin time (seconds)	16.00 ± 0.25
Activated partial thromboplastin time (seconds)	42.05 ± 0.63
International normalized ratio	1.29 ± 0.03
Child-Pugh score	7.09 ± 0.17
Model for the end-stage of liver diseases score	5.59 ± 0.47
Hyaluronic acid (ng/mL)	726.87 ± 168.58
Laminin (ng/mL)	141.76 ± 9.49
Amino-terminal propeptide of type III procollagen (ng/mL)	88.94 ± 10.75
Collagen IV (ng/mL)	157.32 ± 13.95
Hyaluronic acid above the reference value: *n*.	117
Laminin above the reference value: *n*	71
Amino-terminal propeptide of type III procollagen above the reference value: *n*	114
Collagen IV above the reference value: *n*	83

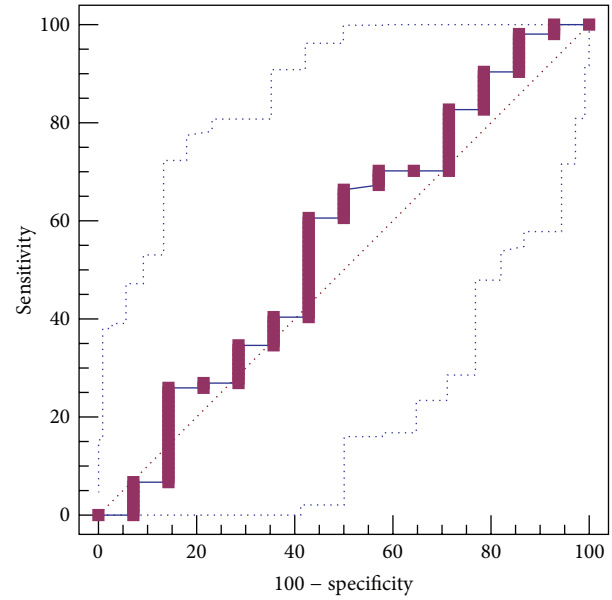

Figure 1: ROC curve analysis to identify the discriminative capacity of HA level in predicting the presence of GEVs in liver cirrhosis.

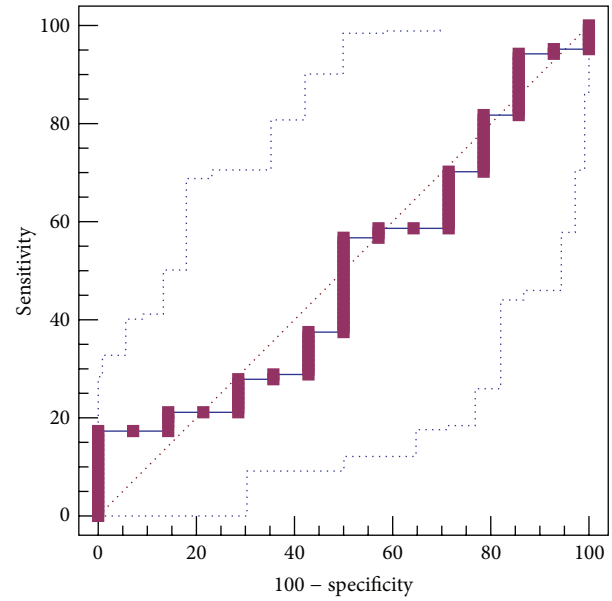

Figure 2: ROC curve analysis to identify the discriminative capacity of LN level in predicting the presence of GEVs in liver cirrhosis.

was 0.490 ± 0.0818 (95% CI: 0.397 to 0.584, $P = 0.9065$) (Figure 2). The best cut-off value was 39.73 with a sensitivity of 17.3% and a specificity of 100%.

PIIINP level was not significantly different between patients with and without GEVs (93.76 ± 12.00 versus 53.16 ± 13.48). The AUROC for PIIINP level in predicting the presence of GEVs was 0.622 ± 0.0762 (95% CI: 0.528 to 0.710, $P = 0.1099$) (Figure 3). The best cut-off value was 31.25 with a sensitivity of 73.1% and a specificity of 57.1%.

CIV level was not significantly different between patients with and without GEVs (153.12 ± 14.47 versus 188.51 ± 48.58). The AUROC for CIV level in predicting the presence of GEVs was 0.560 ± 0.0877 (95% CI: 0.466 to 0.652, $P = 0.4909$) (Figure 4). The best cut-off value was 95.3 with a sensitivity of 39.4% and a specificity of 78.6%.

4. Discussion

The liver fibrosis progression is the major cause for the occurrence and development of portal hypertension in liver cirrhosis. GEVs are one of the most important portal hypertension-related complications. It is reasonable to suppose that the markers of liver fibrosis may reflect the presence and severity of GEVs. However, we would like to emphasize that only very few studies have evaluated the correlation between serum liver fibrosis markers and varices in liver cirrhosis [19–21]. By comparison, the role of liver stiffness measurement in

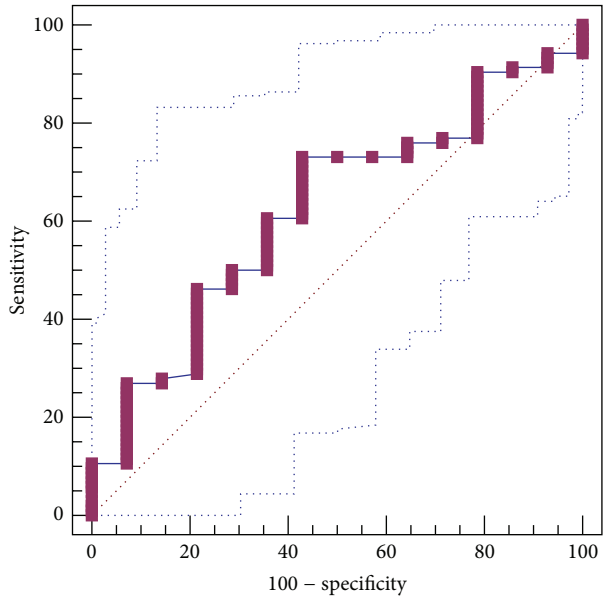

Figure 3: ROC curve analysis to identify the discriminative capacity of PIIINP level in predicting the presence of GEVs in liver cirrhosis.

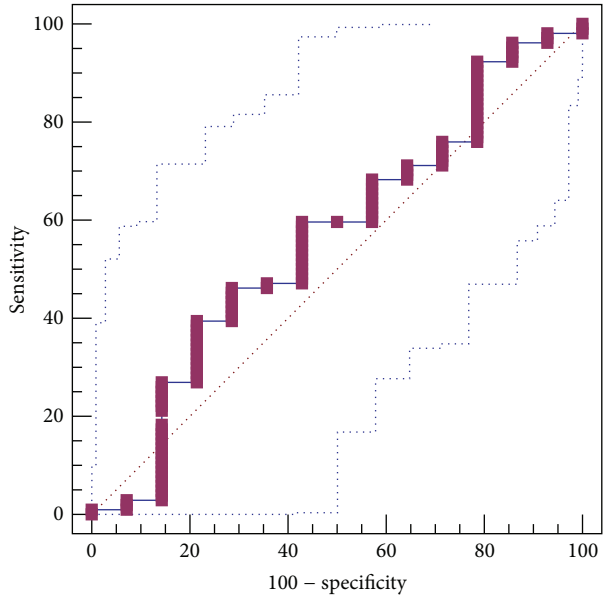

Figure 4: ROC curve analysis to identify the discriminative capacity of CIV level in predicting the presence of GEVs in liver cirrhosis.

predicting varices in liver cirrhosis has been widely discussed. Herein, we attempted to indirectly discuss the possible role of noninvasive diagnostic methods of liver fibrosis by presenting the data from liver stiffness measurement.

Numerous studies and meta-analyses confirmed the significance of liver stiffness measurement for the diagnosis of liver fibrosis. Friedrich-Rust et al. performed a meta-analysis of 50 studies to assess the performance of transient elastography for diagnosing with liver fibrosis [23]. They found that transient elastography carried an excellent diagnostic accuracy with a mean AUROC of 0.84 (95% CI: 0.82 to 0.86) for significant fibrosis, 0.89 (95% CI: 0.88 to 0.91) for severe fibrosis, and 0.94 (95% CI: 0.93 to 0.95) for cirrhosis. Chon et al. also conducted a meta-analysis of 18 studies to evaluate the performance of transient elastography for diagnosing with liver fibrosis in patients with chronic hepatitis B virus infection [24]. Similarly, transient elastography provided a good diagnostic accuracy with a mean AUROC of 0.859 (95% CI: 0.857 to 0.860) for significant fibrosis, 0.887 (95% CI: 0.886 to 0.887) for severe fibrosis, and 0.929 (95% CI: 0.928 to 0.929) for cirrhosis. More recently, Singh et al. performed a meta-analysis of individual participant data from 12 studies and found that magnetic resonance elastography had a high diagnostic accuracy with a mean AUROC of 0.84 (95% CI: 0.76 to 0.92) for any fibrosis, 0.88 (95% CI: 0.84 to 0.91) for significant fibrosis, 0.93 (95% CI: 0.90 to 0.95) for severe fibrosis, and 0.92 (95% CI: 0.90 to 0.94) for cirrhosis [25].

On the other hand, there was a good correlation of liver stiffness with hepatic venous pressure gradient and varices in liver cirrhosis [5, 10, 18, 26]. In the study by Castéra et al., the sensitivity and specificity of transient elastography at a cut-off value of 21.5 kPa in predicting the presence of varices were 76% and 78%, respectively [6]. In the study by Saad et al., liver stiffness measured by FibroScan was regarded as the independent predictor of GEVs (odds ratio = 1.113, $P = 0.005$) [7]. In addition, compared with hepatic venous pressure gradient, liver stiffness also had a similar performance for predicting the occurrence of portal hypertension-related complications (AUROC: 0.815 (95% CI: 0.727 to 0.903) versus 0.837 (95% CI: 0.754 to 0.920)) [27].

In accordance with such a good performance of the liver stiffness measurement in assessing both liver fibrosis and GEVs, further studies may be attractive to evaluate the potential utility of serum liver fibrosis markers tests. Indeed, the clinical evidence has demonstrated that serum liver fibrosis markers are useful in diagnosing the presence of liver fibrosis and classifying its severity [28, 29]. However, it remains unclear whether or not serum liver fibrosis markers can be also useful for screening the presence of GEVs.

Our study did not confirm any significant predictive values of HA, LN, PIIINP, and CIV levels in the assessment of GEVs in liver cirrhosis. This unexpected finding could be explained by a high proportion of patients with history of upper gastrointestinal bleeding and a small number of patients without GEVs ($n = 14$), which might result in the instability of their statistical results. The imbalance in the number of observed patients between the two groups might be primarily attributed to the bias of patient selection. Indeed, only in-patients were enrolled in our study, suggesting that most of our cases had relatively severe conditions or were at decompensated stage. Additionally, serum liver fibrosis markers are more likely to reflect the fibrogenesis, rather than the status of fibrosis. They correlate with the activity of liver diseases (i.e., active alcohol abuse or viral replication). Serum liver fibrosis markers become normal, if liver disease is inactive. Accordingly, it may be less likely to be in a good correlation of serum liver fibrosis markers with portal hypertension.

In spite of the fact that there is no statistical significance, the detailed information should be mentioned. (1) PIIINP level was higher in patients with GEVs than in those without. (2) The mean AUROC for PIIINP was 0.622. (3) P value was close to 0.05. Thus, well-designed studies with appropriate selection of out-patients or patients at compensated stage should validate the role of PIIINP in predicting the presence of GEVs in liver cirrhosis.

In conclusion, based on this retrospective study, the HA, LN, PIIINP, and CIV levels cannot be recommended to evaluate the presence of GEVs in liver cirrhosis. Certainly, the potential utility of PIIINP should be further confirmed in prospective studies. Additionally, given the retrospective nature of this study, the severity and location of GEVs were not clearly evaluated in all patients according to the medical records and endoscopy reports. Accordingly, further studies might be warranted to identify the role of serum liver fibrosis in predicting the presence of large varices or gastric/esophageal varices in liver cirrhosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 357360, 8 pages
http://dx.doi.org/10.1155/2015/357360

Research Article

Assessing the Effect of Leptin on Liver Damage in Case of Hepatic Injury Associated with Paracetamol Poisoning

Murat Polat,[1] Serkan Cerrah,[1] Bulent Albayrak,[1] Serkan Ipek,[2] Mahmut Arabul,[2] Fatih Aslan,[2] and Omer Yilmaz[3]

[1]*Department of Internal Medicine, Erzurum Ataturk University, Yakutiye, 25240 Erzurum, Turkey*
[2]*Department of Gastroenterology, Erzurum Ataturk University, Yakutiye, 25240 Erzurum, Turkey*
[3]*Department of Gastroenterology, Atatürk Training and Research Hospital, İzmir Katip Çelebi University, Karabaglar, 35360 İzmir, Turkey*

Correspondence should be addressed to Mahmut Arabul; mahmutarabul@gmail.com

Received 18 March 2015; Accepted 24 May 2015

Academic Editor: Marcela Kopacova

Background Aim. In case of high-dose acetaminophen intake, the active metabolite can not bind to the glutathione, thereby inducing cellular necrosis through binding to the cytosol proteins. This trial was performed to histologically and biochemically investigate whether leptin was protective against liver damage induced by paracetamol at toxic doses. *Material and Method*. In our trial, 30 female rats, divided into 5 groups, were used. IP leptin administration was performed after an hour in the group of rats, in which paracetamol poisoning was induced. The groups were as follows: Group 1: the control group, Group 2: 20 μg/kg leptin, Group 3: 2 g/kg paracetamol, Group 4: 2 g/kg paracetamol + 10 μg/kg leptin, and Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. *Results*. The most significant increase was observed in the PARA 2 g/kg group, while the best improvement among the treatment groups occurred in the PARA 2 g/kg + LEP 10 μg/kg group ($p < 0.05$). While the most significant glutathione (GSH) reduction was observed in the PARA 2 g/kg group, the best improvement was in the PARA 2 g/kg + LEP 10 μg/kg group ($p < 0.05$). *Conclusion*. Liver damage occurring upon paracetamol poisoning manifests with hepatocyte breakdown occurring as a result of inflammation and oxidative stress. Leptin can prevent this damage thanks to its antioxidant and anti-inflammatory efficacy.

1. Introduction

It is known for a long time that high doses of acetaminophen lead to hepatic and renal damage and event death. While the exact mechanism of hepatorenal damage remains unknown, oxidative stress is mentioned most commonly. Acetaminophen is metabolized to its toxic reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). In case of high-dose acetaminophen intake, the active metabolite cannot bind to the glutathione, thereby inducing cellular necrosis through binding to the cytosol proteins [1].

Leptin is the 167-aminoacid hormonal protein product of the obesity gene, which has been investigated thoroughly after being discovered [2]. Leptin, initially described as being associated with being fuel and the energy balance, was suggested to be an antiobesity factor with a feedback effect on the hypothalamus following release from the adipocytes. On the other hand, the study results show that leptin is involved in various physiologic events such as the regulation of metabolism, sexual development, and reproduction [3]. Leptin is known to have a significant role in innate and acquired immunity. The increase of the leptin levels during infection/inflammation suggests that it is a significant factor involved in the host's response to inflammation. Anorexia, observed during the course of the infections, is believed to be the acute phase response of the host. Bacteria/virus products also stimulate the production of the proinflammatory cytokines (interleukins, TNF-alpha, and interferons). Cytokines increase the leptin expression in the fatty tissue. Both microbic products and the resulting cytokines and leptin reduce food intake. Therefore, particularly, TNF-alpha, interleukin-1 (IL-1), and IL-6 are considered to be responsible for inflammation and anorexia occurring during infection and leptin is considered to partially mediate these effects of

cytokines [4]. Apart from its various metabolic effects, studies suggest that leptin may also be involved in the regulation of the oxidant/antioxidant balance. Some in vivo and in vitro trials have shown that leptin deficiency was associated with the deficiency of antioxidants. Furthermore, there are findings demonstrating that systemic leptin administration could increase the antioxidant efficacy, deemed to be inadequate in the plasma of people with leptin gene mutation and ob/ob mice with leptin deficiency [5]. Growing evidence underlines the significance of leptin, a hormone that is very important in the regulation of the body weight and food intake both in animals and humans [6]. This trial was performed to histologically and biochemically investigate whether leptin was protective against liver damage induced by paracetamol at toxic doses.

2. Material and Method

In our trial, 30 female rats, divided into 5 groups, were used. IP leptin administration was performed after an hour in the group of rats, in which paracetamol poisoning was induced. The groups were as follows: Group 1: the control group, Group 2: 20 μg/kg leptin, Group 3: 2 g/kg paracetamol, Group 4: 2 g/kg paracetamol + 10 μg/kg leptin, and Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. The trial was terminated 24 hours after paracetamol administration and biochemical and histopathological investigations were performed on the tissue and blood samples obtained from the animals.

2.1. Experimental Animals. 30 Albino Wistar rats, weighing between 195 and 205 grams, provided from the experimental animal laboratory of the Ataturk University Experimental Research and Application Center, were used in the trial. The rats were fed and watered ad libitum (Feed Institution Standard Rat Feed). The animals were kept and fed at room temperature (22°C) in groups at the laboratory prior to the trial. The study's compliance with the ethical rules was approved by the "Ataturk University Animal Experiments Local Ethics Committee."

2.2. Pharmaceuticals and Chemical Substances. Paracetamol (Doğa İlaç Hammaddeleri Ticaret Ltd. Şti.) was used in the study after 2 grams was dissolved in 1% CMC (carboxymethyl cellulose) in PBS (phosphate buffer solution) and mixed under slight heat. It was administered to the rats via IP route.

Leptin, L5037-1 mg (Sigma Aldrich) recombinant, expressed in *E. coli*, was lyophilized; 10 cc was dissolved with 1x PBS. 10 μg/kg and 20 μg/kg doses were administered to the rats via IP route.

Thiopental sodium (IE Ulagay), 50 mg/kg, was administered via IP route for euthanasia.

2.3. Analyses of the Liver Tissue. Following the macroscopic analyses, the rat tissues were stored at −80°C. 100 mg tissue from each rat was homogenized in specific homogenate buffer (in appropriate buffer) on ice using Ultra-Turrax and subsequently centrifuged according to the relevant directives. For the biochemical studies, the malondialdehyde (MDA) levels and the glutathione (GSH) levels from each supernatant were measured, respectively, using the high-sensitivity Cell Biolabs OxiSelect TBARS Assay Kit (MDA Quantitation) STA-330 and Cell Biolabs OxiSelect Total Glutathione (GSSG/GSH) Assay Kit STA-312 ELISA kits, specifically designed for the rat tissue in duplicate for each rat liver. In addition, all data were expressed as mean ± standard deviation per mg of protein in all liver supernatants, homogenized with appropriate buffer.

2.3.1. Protein Assay. The protein concentrations were determined via the Lowry method using the commercial protein standards (Sigma Aldrich, Total protein kit-TP0300-1KT, USA).

2.3.2. Serum Analyses. TNF-alpha measurement: blood transferred into the EDTA-containing biochemical tubes was centrifuged at 4000 rpm for 10 minutes at +4°C. The samples were stored at −80°C until the analysis. The TNF-alpha levels of each sample were measured using a high-sensitivity ELISA kit in duplicate (Invitrogen-KRC3011-USA).

2.4. AST and ALT Measurement. Blood transferred into the EDTA-containing biochemical tubes was centrifuged at 4000 rpm for 10 minutes at +4°C. The samples were stored at −86°C until the analysis. The AST and ALT levels of each sample were measured using a high-sensitivity ELISA kit in duplicate (USCN Life Science-E90207Ra, E91214Ra (China)).

2.5. Histological Analysis. The liver tissues obtained from the rats in all groups were coded and placed in 4% formaldehyde-containing bottles. They were kept in alcohol series at increasing concentrations, then cleaned with xylene, and subsequently kept in paraffin beads in the oven. Then the tissues were embedded in paraffin blocks and prepared for the sectioning procedure. 4-μm sections cut from the paraffin blocks using a microtome (Leica Dsc, Germany) were transferred onto the slides and stained with hematoxylin and eosin (Merck©, Germany). Sections, made ready for investigation, were investigated under a light microscope with an Olympus BH 40 camera attached and photos taken for all relevant groups.

2.6. Statistical Analysis. The statistical assessments of the histological studies were performed using the SPSS 18.0 software. The numeric intensity of the necrotic cells was calculated. The data were expressed as mean ± standard deviation. The one-way variance analysis (ANOVA) test and the "Tukey test" were used for the matched experimental groups in the statistical analysis. Values below $p < 0.05$ were considered to be significant.

The statistical analysis of the biochemical studies was conducted using the SPSS 18.0 software. The parametric data were analyzed via one-way variance analysis (ANOVA) test and the "Duncan" technique, a post hoc test. The values obtained were expressed as mean ± standard deviation and p values below 0.05 were considered to be statistically significant.

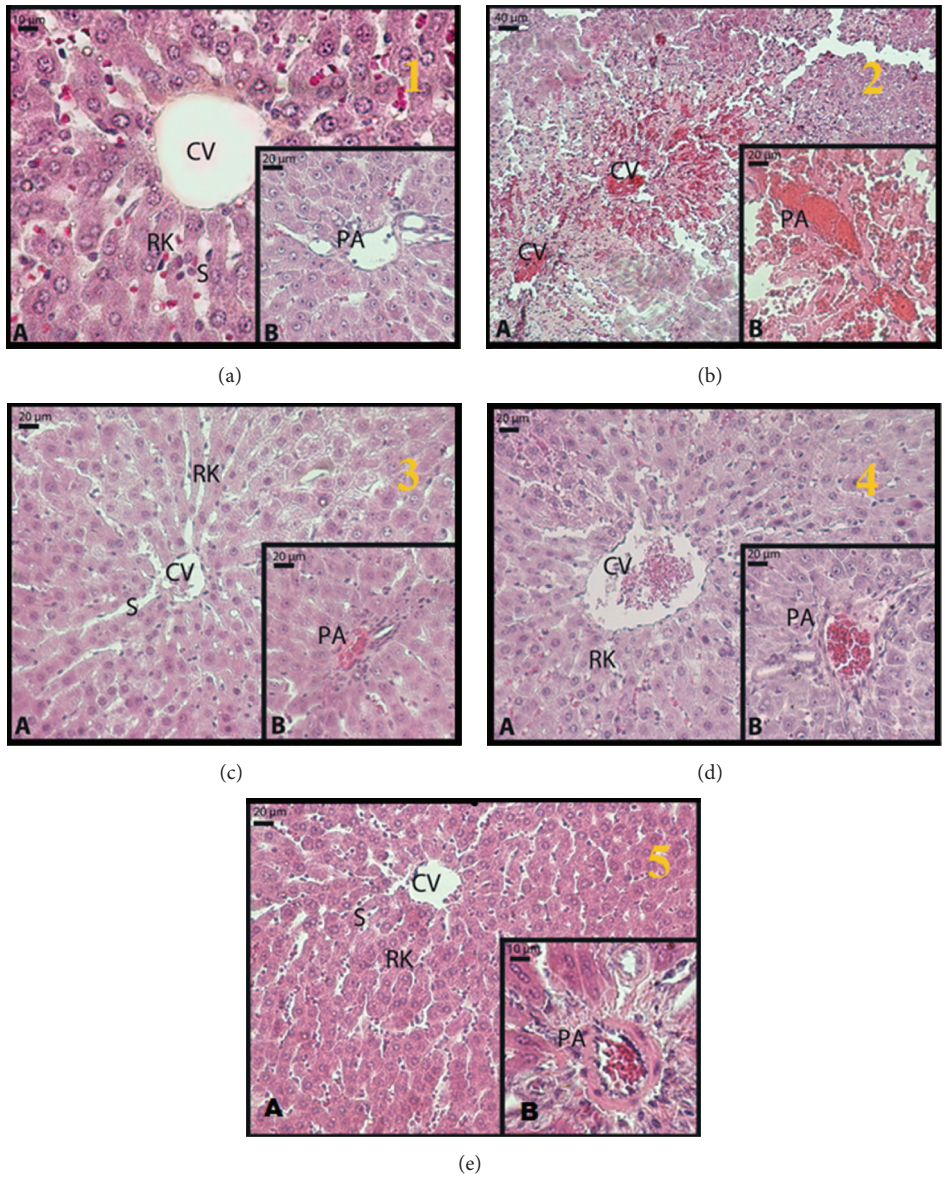

Figure 1: The light microscope images of specimens obtained from the liver samples. (a) Group 1: control group; (b) Group 3: 2 g/kg paracetamol administered group; (c) Group 4: 2 g/kg paracetamol + 10 μg/kg leptin administered group; (d) Group 5: 2 g/kg paracetamol + 20 μg/kg leptin administered group; (e) Group 2: 20 μg/kg leptin administered group (hematoxylin and eosin) (CV: vena centralis, RK: Remark cord, S: sinusoid, PA: portal area).

3. Results

3.1. Histological Results. Investigating the hepatocytes in the parenchyma forming the liver lobules of the experimental animals, the structure was observed to be normal in the control group; that is, these cell cordons, lined up in a radier fashion from the vena centralis to the peripheral lobules (Remark plaques), were recorded as data indicating a normal structure (Figure 1(a), A). Observing the sinusoids, located among the Remark cordons, no structural deformation was detected (Figure 1(a), A and B). The portal regions revealed no abnormality of the vascular structures (Figure 1(a), B).

There were intense necrotic foci in the liver tissue of the animals, which were given paracetamol (Figure 1(b), A). An intense increase of eosinophils in a large number of hepatocytes in the parenchyma, severe hyperchromasia in the nucleus, and irregularity of the cell membrane were observed. Irregular Remark cordons, dilated sinusoids, and intense erythrocyte aggregation were remarkable findings (Figure 1(b), A). In addition, the presence of congestion in both the central vein and the portal area was detected (Figure 1(b), A and B).

Examining the livers of the treated experimental animals, leptin was observed to significantly prevent paracetamol toxicity significantly in the liver. In both 10 μg/kg leptin and 20 μg/kg leptin group, the lining of the hepatocyte cordons from the vena centralis in the parenchyma was quite regular and almost similar to that in the control

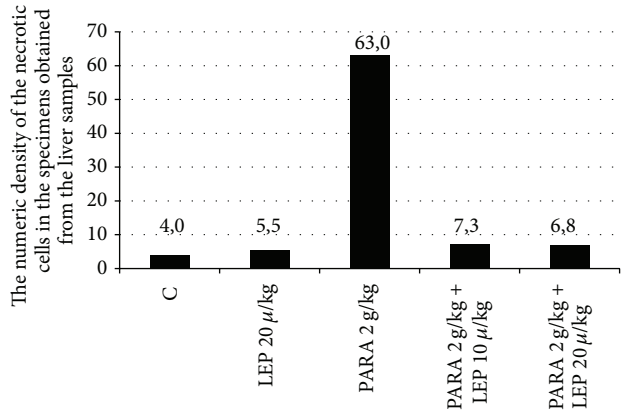

Figure 2: The numeric density of the necrotic cells in the specimens obtained from the liver samples. Group 1: the control group; Group 2: 20 μg/kg leptin; Group 3: 2 g/kg paracetamol; Group 4: 2 g/kg paracetamol + 10 μg/kg leptin; Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. C: control group, LEP: the group receiving leptin, PARA: the group receiving paracetamol. The results were analyzed using the Tukey's technique in the one-way ANOVA test. $p < 0.05$ was considered significant.

Table 1: The numeric density of the necrotic cells in the specimens obtained from the liver samples.

Group	Numeric density of the necrotic hepatocytes (cm^2)
Control	$4 \pm 1.41^{a.b}$
LEP 20 μg/kg	$5.5 \pm 1.87^{b.c}$
PARA 2 g/kg	$63 \pm 16.26^{a.c}$
PARA 2 g/kg + LEP 10 μg/kg	$7.3 \pm 1.21^{b.c}$
PARA 2 g/kg + LEP 20 μg/kg	$6.83 \pm 2.48^{b.c}$

Group 1: the control group; Group 2: 20 μg/kg leptin; Group 3: 2 g/kg paracetamol; Group 4: 2 g/kg paracetamol + 10 μg/kg leptin; Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. LEP: the group receiving leptin, PARA: the group receiving paracetamol. [a] $p < 0.05$ comparison of the control group and paracetamol group, [b] $p < 0.05$ comparison of the control group and treatment group, and [c] $p < 0.05$ comparison of the paracetamol group and the treatment group. $p < 0.05$ was considered significant.

group (Figure 1(d), A and B). In addition, while the vascular degenerations and congestions occurring in the group with induced paracetamol toxicity almost completely recovered (Figure 1(c), A and B), they were slightly more distinct in the 20 μg/kg leptin group relative to the 10 μg/kg leptin group (Figure 1(d), A and B). However, this distinction was not at a level exceeding the physiologic limits.

The other remarkable regularity in the leptin groups was observed in the control group that was administered 20 μg/kg leptin. Data of this group was in line with that from the control group and the group which was administered 20 μg/kg leptin following paracetamol toxicity (Figure 1(e), A and B).

Making a brief comparison between the doses, we can say that the liver structures were more regular in the 10 μg/kg leptin group compared to the 20 μg/kg leptin group.

As can be seen in Table 1 and Figure 2, in comparison to the control group, the number of the necrotic cells was significantly increased only in the paracetamol group ($p \leq 0.001$). The number of the necrotic cells identified in the liver tissues of the groups treated with 10 μg/kg and 20 μg/kg leptin was observed to be significantly decreased compared to the paracetamol group ($p \leq 0.01$). We found a similarity between the leptin 10 μg/kg ($p = 0.663$) and leptin 20 μg/kg ($p = 0.780$) groups and the control group in the number of necrotic cell numbers. The control leptin 20 μg/kg group ($p = 0.973$) was also similar to the control group.

3.2. Biochemical Results

3.2.1. AST, ALT and TNF-α Measurements. As can be seen in Table 2 and Figure 3, the ALT, AST, and TNF-alpha values are as follows: control group: 46.54 U/L-88.58 U/L-41.31 pg/mL, LEP 20 μg/kg: 55.25 U/L-87.65 U/L-48.31 pg/mL, PARA 2 g/kg group: 196.14 U/L-236.30 U/L-154.88 pg/mL, PARA 2 g/kg + LEP 10 μg/kg group: 80.75 U/L-117.26 U/L-43.25 pg/mL, and PARA 2 g/kg + LEP 20 μg/kg group: 122.83 U/L-176.86 U/L-50.75 pg/mL.

Reviewing the results, the most significant increase was observed in the PARA 2 g/kg group, while the best improvement among the treatment groups occurred in the PARA 2 g/kg + LEP 10 μg/kg group ($p < 0.05$).

3.2.2. GSH and MDA Measurements. As can be seen in Table 3 and Figure 4, the GSH and MDA levels measured in the liver tissues obtained from the experimental groups are as follows: control group, 3.84-1.37 nmol/mg; LEP 20 μg/kg group, 3.33-1.29 nmol/mg; PARA 2 g/kg group, 1.74-4.04 nmol/mg; PARA 2 g/kg + LEP 10 μg/kg group, 3.35-1.40 nmol/mg; PARA 2 g/kg + LEP 20 μg/kg group, 2.81-1.91 nmol/mg.

While the most significant GSH reduction was observed in the PARA 2 g/kg group, the best improvement was in the PARA 2 g/kg + LEP 10 μg/kg group ($p < 0.05$). While the MDA level was observed to significantly increase in the PARA 2 g/kg group, the best improvement was in the PARA 2 g/kg + LEP 10 μg/kg group among the treatment groups ($p < 0.05$).

4. Discussion

Despite the abundance of trials on paracetamol toxicity, the mechanism of the liver cell damage has not been elucidated yet. Based on the commonly acceptable opinion, this damage process starts upon paracetamol being metabolized to its reactive metabolite, NAPQI. This metabolite first depletes GSH as a result of being increased in the blood followed by binding to various cellular proteins, which also include the mitochondrial proteins. As a result of this process, suppression of the mitochondrial respiration, consumption of the adenosine triphosphate (ATP), and mitochondrial oxidative stress may occur. ATP consumption results in cellular oncotic necrosis in the hepatocytes and the sinusoidal endothelial cells [7].

Aspartate aminotransferase and alanine aminotransferase are the intracellular enzymes of the liver and the most

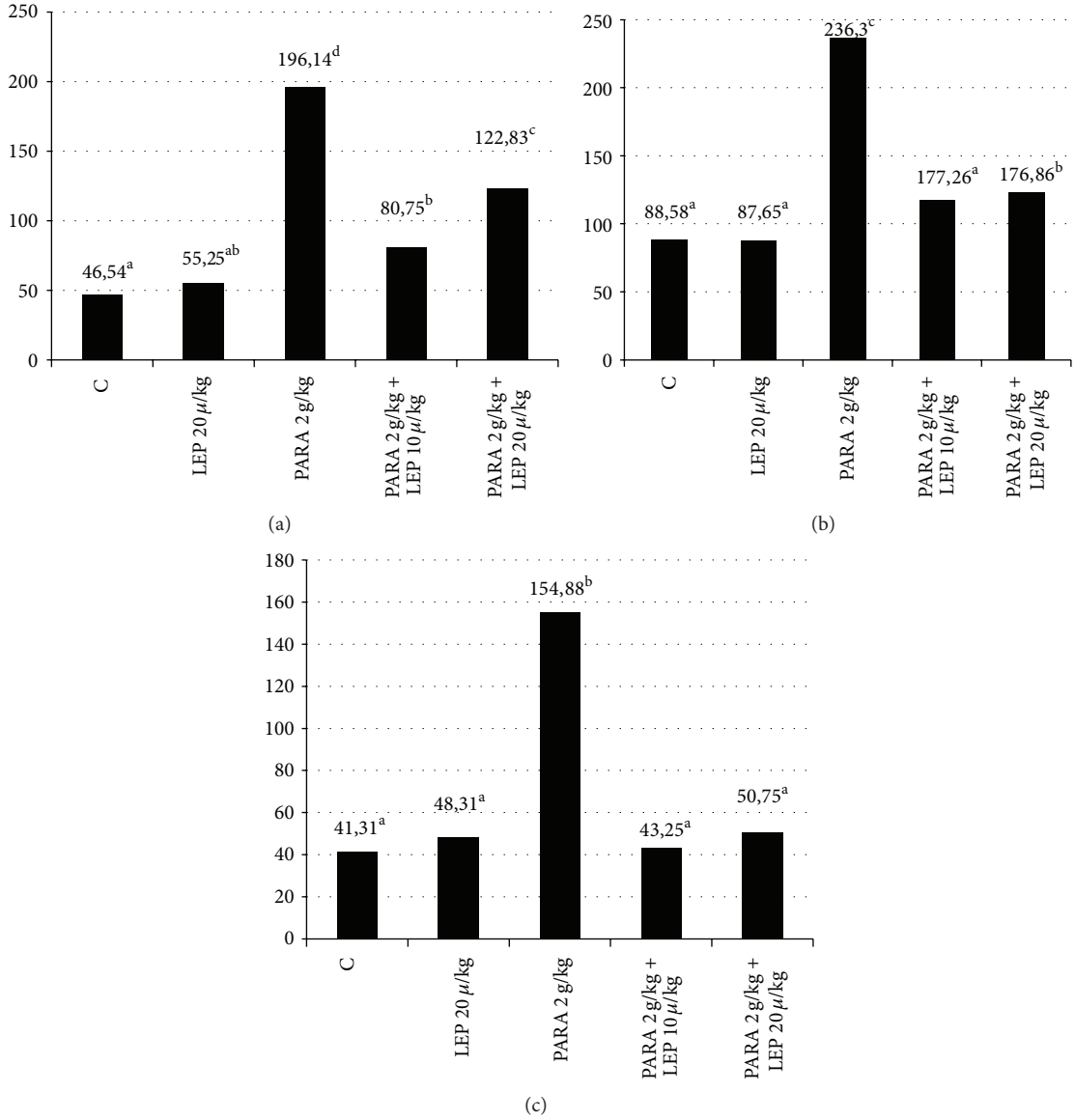

Figure 3: Graphical demonstration of the rat serum ALT level (a); graphical demonstration of the AST level (b); graphical demonstration of the TNF-alpha level (c). Group 1: the control group; Group 2: 20 μg/kg leptin; Group 3: 2 g/kg paracetamol; Group 4: 2 g/kg paracetamol + 10 μg/kg leptin; Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. LEP: the group receiving leptin; PARA: the group receiving paracetamol. There was no statistically significant difference between the values shown with the same letter or letters based on the Duncan multicomparison test. $^{a}p < 0.05$ comparison of the control group and paracetamol group, $^{b}p < 0.05$ comparison of the control group and treatment group, and $^{c}p < 0.05$ comparison of the paracetamol group and the treatment group.

Table 2: ALT and AST levels measured in the rat sera.

Group	ALT (U/L)	AST (U/L)	TNF-α (pg/mL)
Control	46.54 ± 11.67^{a}	88.58 ± 21.03^{a}	41.31 ± 2.36^{a}
LEP 20 μg/kg	$55.25 \pm 13.99^{a,b}$	87.65 ± 11.08^{a}	48.31 ± 9.67^{a}
PARA 2 g/kg	196.14 ± 53.80^{d}	236.30 ± 37.18^{c}	154.88 ± 34.14^{b}
PARA 2 g/kg + LEP 10 μg/kg	80.75 ± 11.67^{b}	117.26 ± 25.31^{a}	43.25 ± 9.53^{a}
PARA 2 g/kg + LEP 20 μg/kg	122.83 ± 39.81^{c}	176.86 ± 57.44^{b}	50.75 ± 12.25^{a}

Group 1: the control group, Group 2: 20 μg/kg leptin. Group 3: 2 g/kg paracetamol. Group 4: 2 g/kg paracetamol + 10 μg/kg leptin. Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. LEP: the group receiving leptin, PARA: the group receiving paracetamol. The results were analyzed using the post-hoc Duncan technique in the one-way ANOVA test. $^{a}p < 0.05$ comparison of the control group and paracetamol group, $^{b}p < 0.05$ comparison of the control group and treatment group, $^{c}p < 0.05$ comparison of the paracetamol group and the treatment group (PARA 2 g/kg + LEP 10 μg/kg), $^{d}p < 0.05$ comparison of the paracetamol group and the treatment group (PARA 2 g/kg + LEP 20 μg/kg), $p < 0.05$ was considered significant.

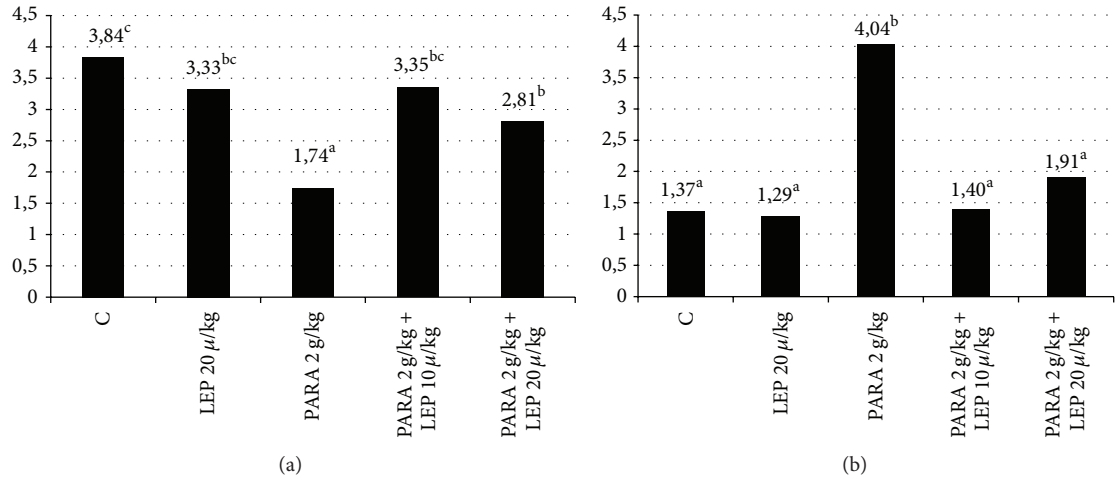

Figure 4: Graphical demonstration of the rat serum GSH level (a); graphical demonstration of the MDA level (b). Group 1: the control group; Group 2: 20 μg/kg leptin; Group 3: 2 g/kg paracetamol; Group 4: 2 g/kg paracetamol + 10 μg/kg leptin; Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. LEP: the group receiving leptin, PARA: the group receiving paracetamol. Malonaldehyde (MDA) levels and the glutathione (GSH). The results were analyzed using the post hoc Duncan technique in the one-way ANOVA test. $^{a}p < 0.05$ comparison of the control group and paracetamol group, $^{b}p < 0.05$ comparison of the control group and treatment group, and $^{c}p < 0.05$ comparison of the paracetamol group and the treatment group. $p < 0.05$ was considered significant.

Table 3: The GSH and MDA levels measured in the rat liver tissues.

Group	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Control	3.84 ± 0.79^{c}	1.37 ± 0.23^{a}
LEP 20 μg/kg	$3.33 \pm 0.72^{b.c}$	1.29 ± 0.76^{a}
PARA 2 g/kg	1.74 ± 0.55^{a}	4.04 ± 1.50^{b}
PARA 2 g/kg + LEP 10 μg/kg	$3.35 \pm 0.71^{b.c}$	1.40 ± 0.78^{a}
PARA 2 g/kg + LEP 20 μg/kg	2.81 ± 0.66^{b}	1.91 ± 0.40^{a}

Group 1: the control group; Group 2: 20 μg/kg leptin; Group 3: 2 g/kg paracetamol; Group 4: 2 g/kg paracetamol + 10 μg/kg leptin; Group 5: 2 g/kg paracetamol + 20 μg/kg leptin. Malondialdehyde (MDA) levels and the Glutathione (GSH). LEP: the group receiving leptin, PARA: the group receiving paracetamol. The results were analyzed using the post hoc Duncan technique in the one-way ANOVA test. $^{a}p < 0.05$ comparison of the control group and paracetamol group, $^{b}p < 0.05$ comparison of the control group and treatment group, and $^{c}p < 0.05$ comparison of the paracetamol group and the treatment group. $p < 0.05$ was considered significant.

reliable two parameters, which increase in case of hepatocellular damage or necrosis [8]. In our trial, we detected a three- to fourfold increase in the AST and ALT values of the group with induced toxicity in the measurements performed on the blood samples obtained from the experimental animals after 24 hours. These results were in compliance with the results obtained from the other trials [9–12]. The AST and ALT values, measured in the groups receiving leptin, were detected to be markedly low relative to the group with induced toxicity. This finding shows that leptin can be enzymatically protective against the liver damage occurring as a result of paracetamol toxicity.

Lipid peroxidation is one of the most significant mechanisms of paracetamol-associated liver damage and is secondary to the free oxygen radicals as a result of oxidative stress. Lipid peroxidation results from the effect of the free radicals on the multi-unsaturated fatty acids. f is the final product of the lipid peroxidation and is a marker, which is commonly used in showing lipid peroxidation. MDA levels increase in tissues, exposed to oxidative stress. This shows that the plasma MDA level can be used as a biomarker for oxidative stress [13]. The MDA value of the liver tissue was detected to be increased in the group with induced paracetamol toxicity and the values obtained were in line with the results from the recent relevant trials [14–16]. In our trial, we detected that the increased oxidative stress could be prevented by leptin. In our trial, we also observed that the blood MDA levels did not significantly increase in rats that were treated with leptin. In line with the results from other trials, this finding showed that leptin created defense against the paracetamol-associated oxidative stress and prevented lipid peroxidation and thus could be effective in maintaining the integrity of the cell membrane [17–19].

Glutathione is one of the most significant antioxidants, which is involved in cellular protection against oxidative stress. GSH is available in the reduced and oxidized forms. Reduced GSH has the ability to give the unstable molecules, such as the reactive oxygen products, the reducing equivalents. The protective effect occurs via this mechanism. In cases such as reduced cellular GSH levels and GSH synthesis capacity, the cells become susceptible to oxidative stress. At the toxic doses of paracetamol, NAPQI, as a mediator of oxidative stress, caused a reduction in the GSH levels and thus hepatic damage [20]. In our trial, the tissue GSH value was observed to be decreased in case of paracetamol-induced hepatotoxicity [21–23]. In our trial, we detected

that the reduction in the GSH values was detected to be statistically significantly avoided in the leptin-treated groups. This finding supports that leptin provides protection against the oxidative stress occurring in the hepatocytes in case of paracetamol poisoning and that it had an antioxidant capacity and reduced cellular damage.

In case of liver necrosis secondary to paracetamol toxicity, serum cytokines and particularly TNF-alpha together with oxidative stress were shown to be involved by the recent trials [24, 25]. TNF-alpha is a proinflammatory cytokine, mostly produced by the macrophages in the liver. It is the primary mediator of systemic toxicity and liver damage. TNF-alpha is known to have a significant role in many forms of liver damage, including ischemia, reperfusion, and fulminant hepatic failure. In addition to initiating the aggressive inflammatory process and deteriorating the cellular damage further, it also acts as a central regulator that stimulates apoptosis and cell proliferation and contributes to tissue repair [26]. In our trial, the TNF-alpha level was observed to be increased 4-fold in the group receiving paracetamol relative to the other groups. This indicates that TNF-alpha significantly increases as a result of the paracetamol toxicity [16, 27, 28]. Our trial results are in compliance with those from the literature trials that show that leptin has an anti-inflammatory efficacy [29, 30] because our leptin groups demonstrated a significant improvement in the TNF-alpha level. This proves that the proinflammatory effects of TNF-alpha, significantly involved in the liver damage, could be prevented by leptin and that exogenous leptin has an anti-inflammatory efficacy.

Liver damage secondary to paracetamol toxicity does not only lead to enzymatic changes but also to histological changes. The use of paracetamol at toxic doses results in the development of centrilobular necrosis [31]. Additionally, at supratherapeutic doses, chronic drug administration was associated with various levels of centrilobular necrosis in the liver and depending on the time and the damage spread to the other regions of the liver lobules [32]. In our trial, intense necrotic foci were observed in line with the other trials performed using paracetamol. Particularly, an intense eosinophil increase in the cytoplasm of a large number of hepatocytes in the parenchyma, severe hyperchromasia in the nucleus, and irregularity of the cell membrane were detected. Irregular Remark cordons, dilated sinusoids, and intense erythrocyte aggregation were remarkable findings. In addition, the presence of congestion in the vascular structures in both the central vein and the portal region was among the other pathological changes observed [11, 12, 21, 33–35]. Reviewing the treatment groups, leptin was observed to significantly prevent paracetamol toxicity in the liver. In both 10 μg/kg leptin and 20 μg/kg leptin groups, the lining of the hepatocyte cordons from the vena centralis in the parenchyma was quite regular and almost similar to that in the control group. Additionally, investigating the hepatocytes closer, no necrotic cells were detected in the group receiving paracetamol; the hepatocytes appeared regular. While the vascular degenerations and congestions occurring in the group with induced paracetamol toxicity almost completely recovered, they were slightly more distinct in the 20 μg/kg leptin group relative to the 10 μg/kg leptin group. However, this distinction was not at a level to exceed the physiologic limits. The similarity of our findings to the results indicating that the substances used in the above trials are protective against paracetamol toxicity shows that leptin has a histologically protective effect against liver damage occurring upon paracetamol poisoning.

In our trial, we administered leptin at the 10 μg/kg and 20 μg/kg doses. The higher level of histological and biochemical improvement observed in the 10 μg/kg leptin group relative to the 20 μg/kg leptin suggests that this dose could be the therapeutic dose of leptin in this indication and that the 20 μg/kg leptin dose could correspond to the supratherapeutic doses.

In conclusion, liver damage occurring upon paracetamol poisoning manifests with hepatocyte breakdown occurring as a result of inflammation and oxidative stress. Leptin can prevent this damage thanks to its antioxidant and anti-inflammatory efficacy. However, further trials are needed to be conducted on this subject.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 936564, 8 pages
http://dx.doi.org/10.1155/2015/936564

Research Article

ATAD2 Overexpression Identifies Colorectal Cancer Patients with Poor Prognosis and Drives Proliferation of Cancer Cells

Yang Luo, Guang-Yao Ye, Shao-Lan Qin, Min-Hao Yu, Yi-Fei Mu, and Ming Zhong

Department of Gastrointestinal Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

Correspondence should be addressed to Ming Zhong; drzhongming@hotmail.com

Received 10 May 2015; Accepted 20 September 2015

Academic Editor: Miroslav Zavoral

ATPase family AAA domain-containing 2 (ATAD2) has been identified as a critical modulator involved in cell proliferation and invasion. The purpose of this study was to explore the expression of ATAD2 in CRC tissues as well as its relationship with degree of malignancy. Data containing three independent investigations from Oncomine database demonstrated that ATAD2 is overexpressed in CRC compared with normal tissue, and similar result was also found in 32 pairs of CRC tissues by qPCR. The protein expression of ATAD2 was examined in six CRC cell lines and 300 CRC specimens. The results showed that high expression of ATAD2 was significantly correlated with tumor size ($P < 0.001$), serum CEA ($P = 0.012$), lymph node metastasis ($P = 0.018$), liver metastasis ($P = 0.025$), and clinical stage ($P = 0.004$). Kaplan-Meier method suggested that higher ATAD2 protein expression significantly associated with the overall survival (OS) of CRC patients ($P < 0.001$) and was an independent predictor of poor OS. Functional studies showed that suppression of ATAD2 expression with siRNA could significantly inhibit the growth in SW480 and HCT116 cells. These results indicated that ATAD2 could serve as a prognostic marker and a therapeutic target for CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most common lethal malignancies in terms of both incidence and mortality [1]. Although the diagnosis and treatment of CRC have been improved, the efficacy of surgery and chemotherapy remains unsatisfactory due to late diagnosis [2]. Therefore, new diagnostic and treatment strategies are urgently needed for this malignancy.

ATPase family AAA domain-containing 2 (ATAD2), also known as ANCCA (AAA+ nuclear coregulator cancer associated), is a novel member of the AAA+ ATPase family [3, 4]. ATAD2 contains both a bromodomain and an ATPase domain and also maps to chromosome 8q24 that is the most commonly amplified region in many types of cancer [5]. The especial structure of ATAD2 indicates that it is associated with genome regulation, including cell proliferation, division, apoptosis, and differentiation [6–10]. Recently, it is reported that aberrant expression of ATAD2 contributes to hepatocellular carcinoma proliferation and metastasis [11, 12]. Studies have revealed that ATAD2 is highly expressed in several types of tumors such as breast cancer, lung cancer, and gastric cancer [13–15]. Thus, ATAD2 manifests oncogenic function and plays a significant role in cancer development. However, the expression of the ATAD2 protein in CRC and its significance remain uncertain.

2. Methods

2.1. In Silico Analysis Using the Oncomine Database. To determine the expression pattern of ATAD2 in CRC, three datasets (Kaiser Colon, Hong Colorectal, and Hong Colorectal) in Oncomine database (https://www.oncomine.org) were used. We compared ATAD2 gene expression in CRC tissues with normal colorectal tissues according to the standard procedures as previously described [16].

2.2. Cell Culture and Transfection. Five human CRC cell lines (HCT116, SW480, LoVo, T84, and HT29) and a normal control colon cell line (HCoEpiC) were all preserved in Shanghai Cancer Institute. All of these cells lines were cultured in DMEM medium (Invitrogen) supplemented with

10% (v/v) fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified incubator under 5% CO_2 condition.

The transfections were performed using Lipofectamine 2000 (Invitrogen, USA). Small interfering RNAs (siRNA) targeting ATAD2 and a negative control were obtained from GenePharma Technology (Shanghai, China). The transfection was performed according to the manufacturer's protocol.

2.3. Patients and Tissue Samples. A total of 300 formalin-fixed paraffin-embedded CRC tissues were collected to perform immunohistochemical staining from January 2005 to November 2014 at the Renji Hospital, Shanghai Jiao Tong University School of Medicine, China. Moreover, additional 32 snap-frozen CRC tissues and corresponding adjacent noncancerous tissues to isolate RNA, which were also obtained from Renji Hospital, were enrolled in this study simultaneously. Inclusion criteria were histologically confirmed CRC and curative resection of tumor without preoperative or postoperative adjuvant therapy. Important clinical data, such as tumor location, serum CEA level, and clinical stage, were collected from each patient's medical records. The follow-up time was calculated from the date of surgery to the date of death, or the last known follow-up. All CRC tissue samples in this study were obtained with patients' written informed consent and all experiments have been approved by the ethics committee at local hospital.

2.4. Real-Time Quantitative PCR. Total RNA from primary tumor and adjacent noncancerous tissue samples was extracted using Trizol reagent (Takara, Japan), and according to the manufacturer's instructions, reverse transcription was performed by PrimeScript RT-PCR kit (Takara, Japan). Real-time quantitative PCR (qPCR) was performed using a 7500 real-time PCR system (Applied Biosystems, Inc., USA). The primers for ATAD2 were as follows: forward: 5′-GGAATCCCAAACCACTGGACA-3′; reverse: 5′-GGTAGCGTCGTCGTAAAGCACA-3′. GAPDH mRNA was used to standardize the relative expression of ATAD2. The primers for GAPDH were as follows: forward: 5′-GCATTGCCCTCAACGACCAC-3′, reverse: 5′-CCACCACCCTGTTGCTGTAG-3′.

2.5. Immunohistochemical Staining. Four-micrometer-thick tissue sections were subjected to immunohistochemical staining with avidin-biotin-peroxidase complex system which was performed as previously described [17]. Tissue sections were incubated by anti-ATAD2 antibody (1 : 400, Abcam, Cambridge, UK) at 4°C overnight. Immunohistochemical staining was scored by two independent pathologists according to intensity and percentage of positive cells simultaneously. Staining intensity was scored as follows: 0: negative; 1: weak staining; 2: moderate staining; 3: strong staining, and the percentage of positive cells was scored on a scale of 0–4 (0, <5%; 1, 5%–30%; 2, 30%–50%; 3, 51%–75%; 4, >75%). And the final score was designated as low or high expression group using the percent of positive cell score × staining intensity score as follows: low expression was defined as a total score < 6 and high expression with a total score ≥ 6.

2.6. Western Blot. Western blot was performed as previously described [18]. Cells were harvested after 48-hour transfection, and 40 μg of protein samples was resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were probed with the same primary antibody used in immunostaining overnight at 4°C and incubated for 1 hour with secondary antibody (Boston, MA, USA). The Western blotting analysis was repeated at least three times.

2.7. Cell Proliferation Assay. Cell viability was assessed by the Cell Counting Kit 8 (CCK-8; Dojindo). Briefly, control and treated SW480 and HCT116 cells were seeded into 96-well plates at an initial density of 3000 cells/well. At each time point, 10 μL of CCK-8 solution was added to each well and incubated for 2 hours. The absorbance was measured by scanning with a microplate reader at 450 nm. The experiment was repeated at least three times.

2.8. Statistical Analysis. Statistical analyses were performed by SPSS 19.0 (SPSS Inc.; Chicago, USA). The expression of ATAD2 mRNA in CRC tissues and corresponding noncancerous tissues was analyzed with the Student's t-test. The differences of the relative absorbance value of CCK8 assays also were determined using Student's t-test. The Chi-square test was used to analyze the relationship between ATAD2 expression and clinicopathological features. Survival rate was evaluated by Kaplan-Meier method and differences between survival curves were tested by the log-rank test. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. ATAD2 Is Overexpressed in CRC at mRNA Level. To roundly investigate ATAD2 expression in CRC, we analyzed three independent microarray datasets from Oncomine database [19–21]. The results showed that the mRNA expression levels of ATAD2 were upregulated in most of CRC tissues, compared with normal tissue (Figures 1(a)–1(c)). Then, 32 pairs of CRC and matched normal tissues were subjected to qPCR. Consistent with the data from Oncomine database, ATAD2 was also overexpressed in 68.75% (22/32) of CRC patients at mRNA level (Figure 1(d)).

3.2. ATAD2 Is Expressed Diversely in CRC at Protein Level. To further investigate the expression of ATAD2 at the protein level, we measured ATAD2 level in CRC cell lines and tissues. The expression of ATAD2 protein was increased in all five CRC cell lines compared with the nonmalignant HCoEpiC cells. Moreover, we found that the higher expression of ATAD2 protein is in low differentiated CRC cell lines (SW480, HCT116, and LoVo) compared with well-differentiated cell lines (HT29, T84) (Figures 1(e)-1(f)). Then, we tested 300 CRC tissue samples by using the method of immunohistochemical staining and found that ATAD2 was low expressed in the 124 (41.33%) of the total 300 CRC samples while the remaining 176 (58.67%) samples remained at a high expression level (Figures 2(a)–2(c)).

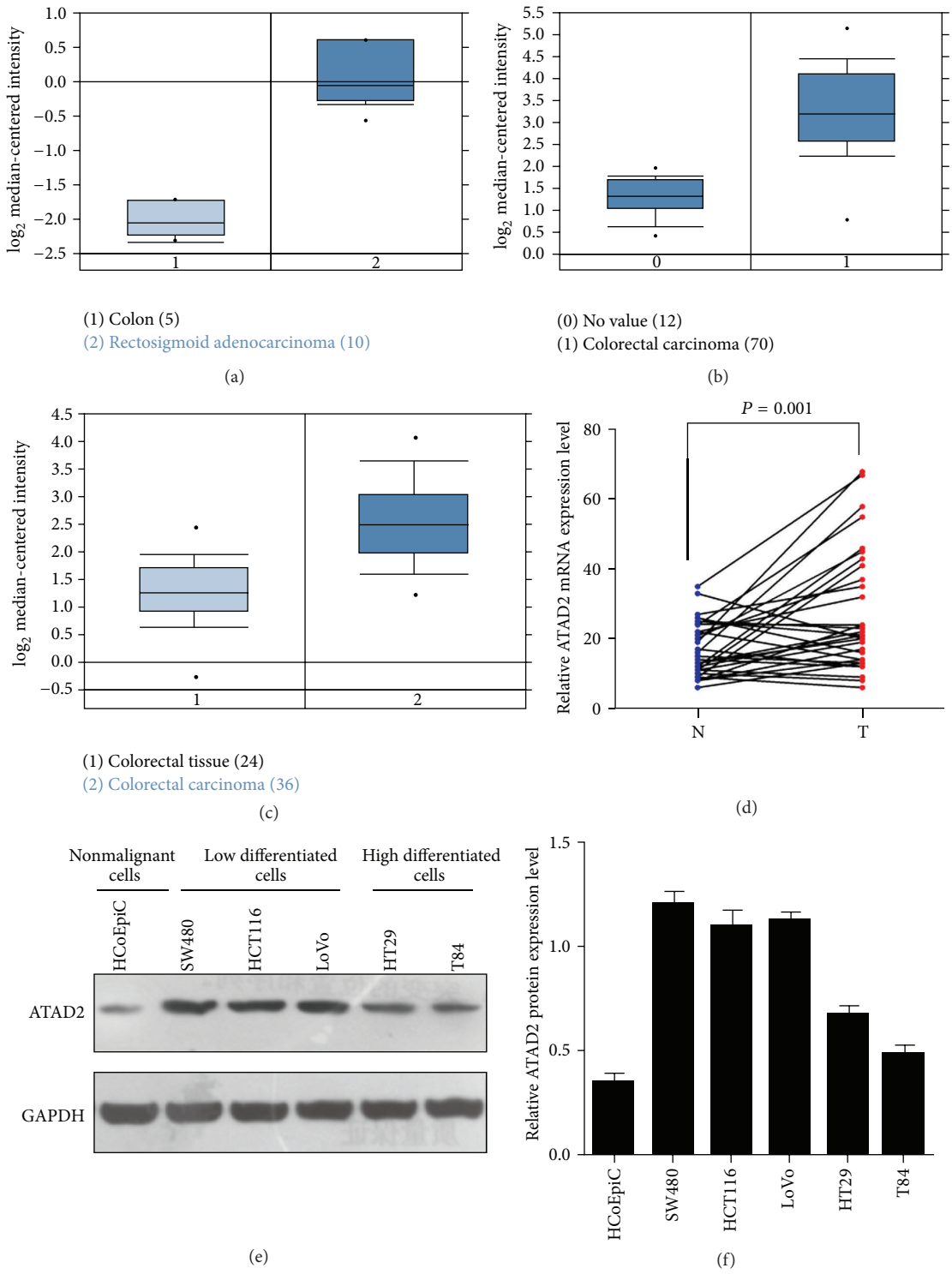

Figure 1: ATAD2 expression in CRC at mRNA and protein level. (a)–(c) ATAD2 expression in Kaiser Colon (a), Hong Colorectal, (b) and Skrzypczak Colorectal (c). (d) Increased ATAD2 mRNA expression in 32 matched tumor (T) and nontumor tissues (N) was detected by qPCR. (e)-(f) Western blots show the ATAD2 expression in five CRC cell lines and the nonmalignant HCoEpiC cells. *P* values were calculated by paired *t*-test.

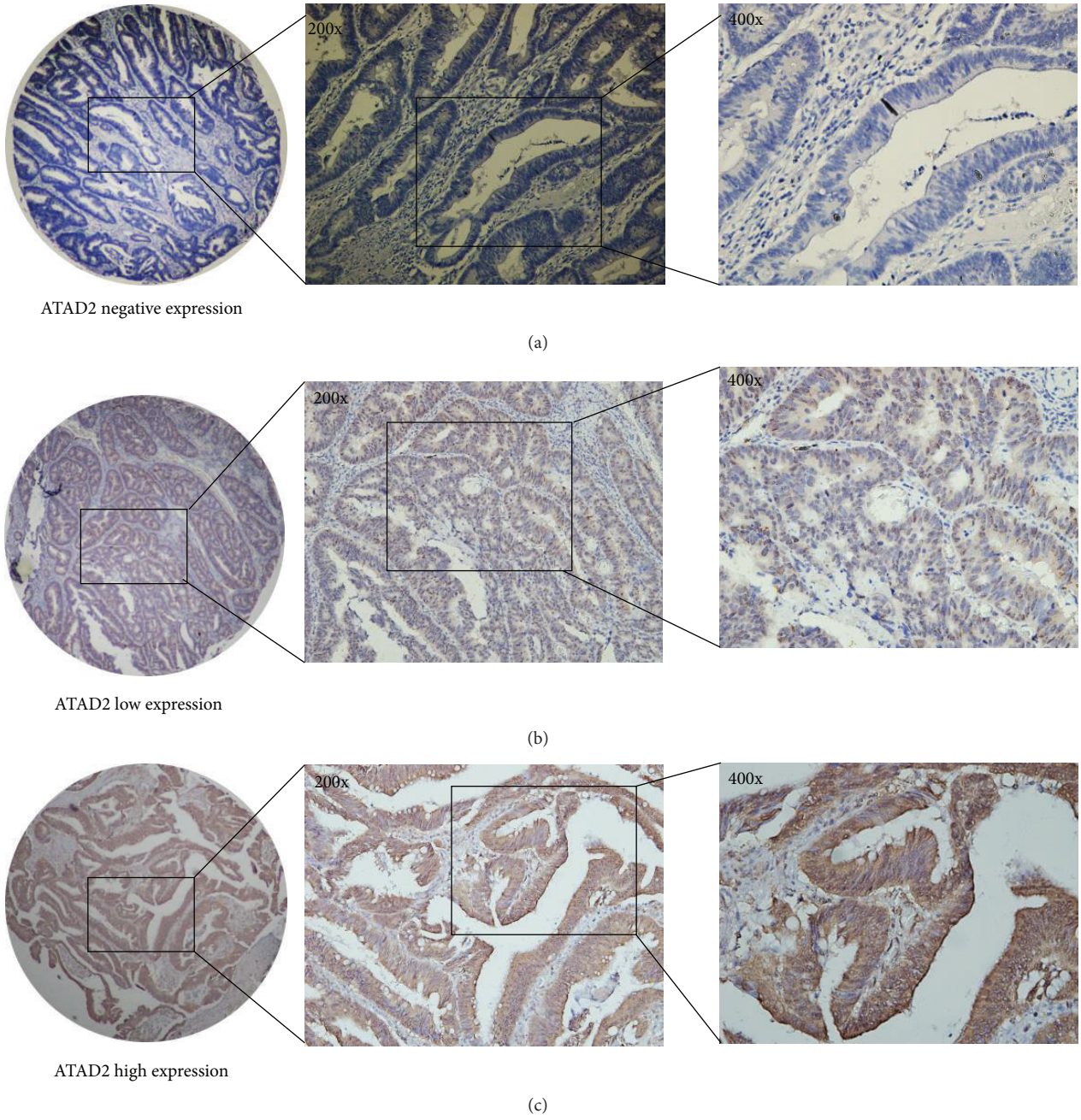

Figure 2: ATAD2 expression in CRC was determined by immunochemistry. (a) Negative expression of ATAD2, (b) low expression of ATAD2, and (c) high expression of ATAD2. Representative images are shown at 200x and 400x magnification, respectively.

3.3. Relationship between ATAD2 Expression and Corresponding Clinical Parameters. To evaluate the clinical significance of ATAD2, the Chi-square test was used to analyze correlations between ATAD2 protein expression and clinicopathological parameters in CRC. The results indicated that high expression of ATAD2 in CRC tissues is closely correlated with tumor size ($P < 0.001$), serum CEA level ($P = 0.012$), lymph node metastasis ($P = 0.018$), liver metastasis ($P = 0.025$), and clinical stage ($P = 0.004$). However, no statistically significant correlations were identified between ATAD2 expression and other clinicopathologic characteristics, including age, gender, and tumor location (Table 1).

3.4. Correlation between ATAD2 Expression and Prognosis in CRC Patients. To investigate the prognostic influence of ATAD2, the overall survival (OS) rate of CRC patients was analyzed using Kaplan-Meier survival curves and the log-rank test. The result revealed that high expression of ATAD2 was inversely associated with OS for all 189 samples ($P < 0.001$) (Figure 3(a)). In addition, The OS of ATAD2 negative

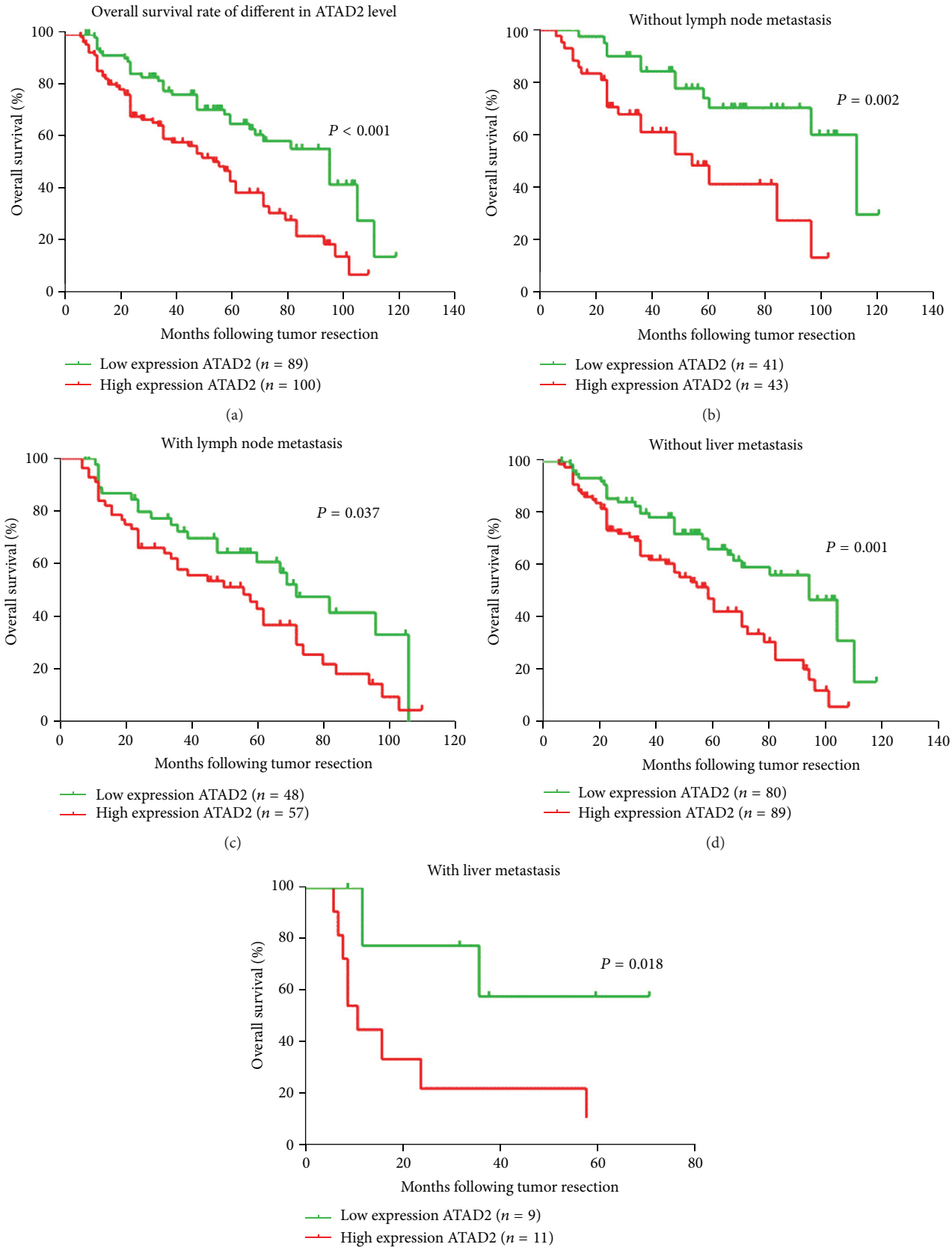

Figure 3: ATAD2 is correlated with overall survival rate in CRC patients. (a) Kaplan-Meier survival curves show high expression level of ATAD2 is significantly correlated with poor survival of CRC. (b)–(e) Correlation between ATAD2 expression and patient survival in colorectal cancer is independent of lymphatic metastasis and liver metastasis. *P* values were calculated using the log-rank test.

Table 1: Relationship between ATAD2 expression and clinicopathological features in 300 colorectal cancer patients.

Variable	ATAD (n) Low $n = 124$	ATAD (n) High $n = 176$	P
Age			
≤65 years	73 (24.33)	97 (32.33)	0.518
>65 years	51 (17.00)	79 (26.34)	
Gender			
Male	68 (22.37)	99 (32.57)	0.978
Female	56 (18.42)	81 (26.64)	
Tumor size			
≤5 cm	78 (26.00)	72 (24.00)	**<0.001**
>5 cm	46 (15.33)	104 (34.67)	
Tumor location			
Rectum	73 (24.33)	103 (34.33)	0.873
Colon	51 (17.01)	73 (24.33)	
Serum CEA			
≤5 ng/mL	74 (24.67)	79 (26.33)	**0.012**
>5 ng/mL	50 (16.67)	97 (32.33)	
Lymph node metastasis			
N0	53 (17.67)	52 (17.33)	**0.018**
N1-N2	71 (23.67)	124 (41.33)	
Liver metastasis			
M0	109 (36.33)	137 (45.66)	**0.025**
M1	15 (5.00)	39 (13.00)	
Clinical stage			
I	17 (5.67)	12 (4.00)	
II	23 (7.67)	16 (5.33)	**0.004**
III	69 (23.00)	109 (36.33)	
IV	15 (5.00)	39 (13.00)	

group was distinctly better than that of the ATAD2 positive one for samples separated according to lymphatic metastasis and liver metastasis (Figures 3(b)–3(e)).

Furthermore, univariate and multivariate analyses were performed to confirm the possibility of ATAD2 used as an independent risk factor for poor prognosis in the 182 cases of CRC. Univariate Cox regression analyses showed that ATAD2 expression, tumor size, serum CEA, liver metastasis, and clinical stage were significantly associated with overall survival (OS) (Table 2). The multivariate Cox regression analysis confirmed ATAD2 expression, tumor size, and clinical stage as independent predictors of the OS in CRC patients (Table 2).

3.5. Effect of ATAD2 on CRC Cell Proliferation In Vitro. To better understand the biological function of ATAD2, we transfected siRNAs-ATAD2 into SW480 and HCT116 cells, and the expression levels of endogenous ATAD2 proteins were significantly suppressed by Western blot (Figure 4(a)). Moreover, we found that knockdown of ATAD2 resulted in a significant decrease in cell viability measured by CCK-8, compared with control (Figures 4(b)-4(c)).

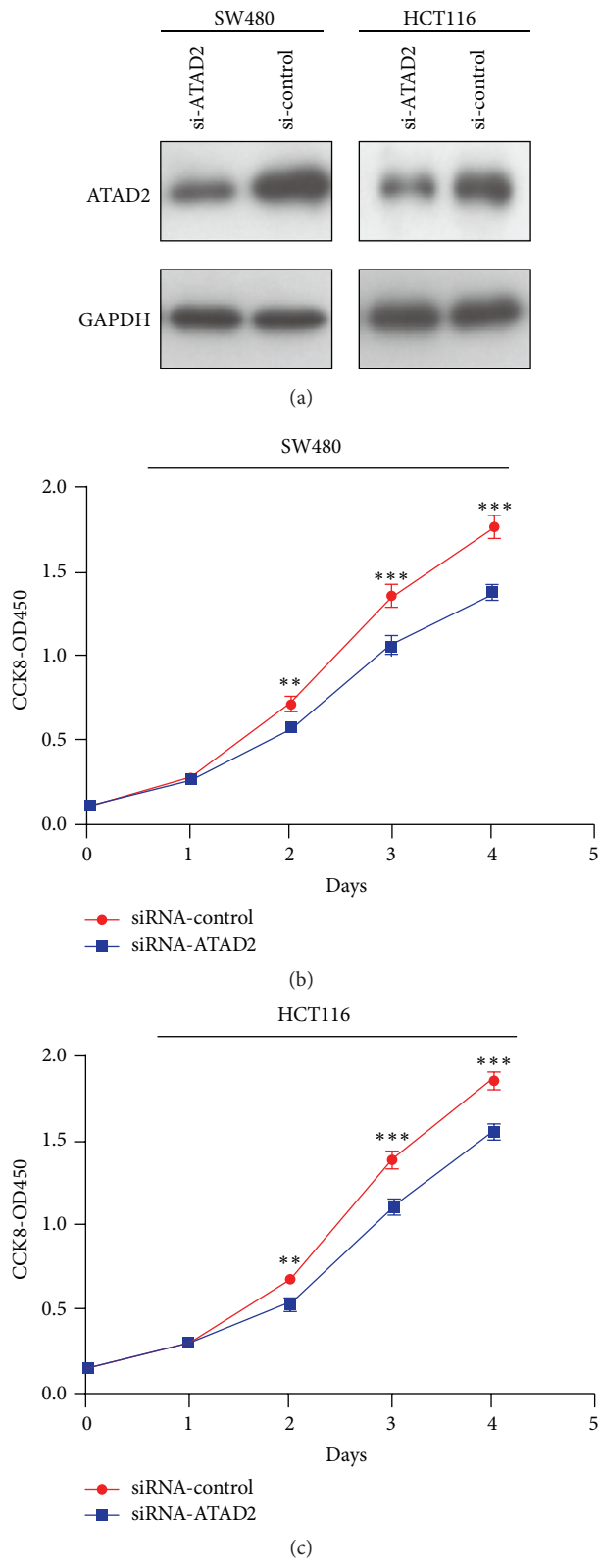

Figure 4: Effect of siRNA-ATAD2 on the proliferation of SW480 and HCT116 cells. (a) ATAD2 knockdown efficiency was confirmed by qPCR in SW480 and HCT116 cells. (b)-(c) siRNA-ATAD2 decreased cell viability measured by CCK8 assays. P values were calculated by Student's t-test ($^{**}P < 0.01$, $^{***}P < 0.001$) and the experiment was repeated at least three times.

Table 2: Univariate and multivariate analyses showing the overall survival in colorectal cancer.

Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
ATAD2 (high versus low)	2.272	1.479–3.491	**0.000**	1.762	1.113–2.790	**0.016**
Age (>65 versus ≤65)	1.393	0.911–2.132	0.127	—	—	—
Gender (male versus female)	1.362	0.905–2.048	0.139	—	—	—
Tumor size (>5 cm versus ≤5 cm)	1.842	1.201–2.825	**0.005**	1.698	1.075–2.681	**0.023**
Tumor location (colon versus rectum)	0.770	0.497–1.194	0.243	—	—	—
Serum CEA (>5 ng/mL versus ≤5 ng/mL)	1.628	1.063–2.493	**0.025**	1.497	0.979–2.291	0.063
Lymph node metastasis (present versus absent)	1.686	1.099–2.588	**0.017**	1.038	0.644–1.673	0.879
Liver metastasis (present versus absent)	3.308	1.777–6.151	**0.000**	1.710	0.777–3.764	0.183
Clinical stage (I vs. II vs. III vs. IV)	1.983	1.549–2.539	**0.000**	1.611	1.168–2.221	**0.004**

HR: hazard ratio; CI: confidence interval. The bold number represents the *P* values with significant differences.

4. Discussion

Studies focused on human clinical specimens and genetically engineered mouse models of CRC have led to a better understanding of this genetic malignancy [22]. ATAD2 through different mechanisms broadly participated in various tumor types, such as prostate cancer, lung cancer, breast cancer, and ovarian cancer [4, 13, 14, 23]. Previous studies indicated that ATAD2 directly interacted with the oncogene AIB1/ACTR and played an important role in the recruitment of ERα to promote the expression of genes driving cancer cell proliferation [24]. Furthermore, the ATPase domain of ATAD2 also enhanced the E2 induction of cyclin D1 and E2F1 expression [4]. In addition, ATAD2 was also directly associated with AR to activate AR-mediated transcription and was required to regulate the expression of androgen-induced genes that controlled cancer cell proliferation and survival [4].

In the current study, we firstly assessed ATAD2 expression at mRNA level. Both data from Oncomine database and our results showed that most primary CRC tissues exhibited significantly higher mRNA expression of ATAD2 than their matched normal tissues. This tendency was then confirmed by immunohistochemistry where 176 of 300 (58.67%) CRC tissues were found to have high protein expression of ATAD2. Previous studies had demonstrated that overexpression of ATAD2 at the transcriptional level via miR-372 regulation promotes tumor malignancy and consequently results in poor outcome in liver adenocarcinoma patients [11]. Our results revealed similar phenomenon that ATAD2 has positive correlation with serum CEA level, lymph node metastasis, distant metastasis, and clinical stage in CRC. Besides, patients with a high level of ATAD2 expression had significantly shorter survival times compared to those with a low level of ATAD2 expression. Furthermore, univariate analysis showed that elevated ATAD2 expression was significantly associated with the OS of CRC patients; multivariate analysis demonstrated that ATAD2 expression, tumor size, and clinical stage are independent risk factors for the prognosis of CRC patients. Collectively, these results suggest that ATAD2 may be involved in the initiation and progression of CRC. Finally, we found that downexpression of ATAD2 could dramatically suppress the proliferation of SW480 and HCT116 cells, which is in keeping with the previous studies on the other cancer cells. However, its underlying molecular mechanisms need to be further investigated.

In conclusion, our study demonstrated that ATAD2 expression was increased in CRC tissues compared to adjacent normal tissues and might be associated with pathological development of CRC. In addition, we found that high expression of ATAD2 could serve as an independent prognostic factor for CRC patients. Therefore, ATAD2 may be an important clinical marker of therapy for CRC.

Conflict of Interests

The authors declared that they have no competing interests.

Authors' Contribution

Yang Luo, Guang-Yao Ye, and Shao-Lan Qin contributed equally to this work.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 937682, 7 pages
http://dx.doi.org/10.1155/2015/937682

Research Article

Prognostic Value of Metastatic No.8p LNs in Patients with Gastric Cancer

Dong-Jiao Guo, Kun Yang, Wei-Han Zhang, Xiao-Long Chen, Xin-Zu Chen, Bo Zhang, Zong-Guang Zhou, and Jian-Kun Hu

Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

Correspondence should be addressed to Jian-Kun Hu; hujkwch@126.com

Received 14 December 2014; Revised 29 March 2015; Accepted 9 April 2015

Academic Editor: Zuzana Zelinkova

Background. To evaluate prognostic value of metastatic No.8p LNs in patients with gastric cancer. *Methods*. From August 2002 to December 2011, a total of 284 gastric cancer patients who underwent gastrectomy with No.8p LNs dissection were analyzed retrospectively in this study. Patients were divided into two groups according to the status of No.8p LNs. Clinicopathological features were collected to conduct the correlation analysis. Follow-up was carried out up to December 31st, 2014. Overall survival was analyzed. *Results*. Out of 284 patients, metastatic No.8p LNs were found in 24 (8.5%) patients. Compared with other 260 cases, these patients suffered morphologically larger tumor ($P = 0.003$), node stage ($P = 0.000$), and metastatic stage ($P = 0.000$). The 3-year overall survival rate was 26% in No.8p-positive group and 53% in No.8p-negative group. No significant difference of cumulative survival rates existed between the No.8p-positive group and No.8p-negative stage IV group (26% versus 28%, $P = 0.923$). Patients with other distant metastasis or not in No.8p+ group had similar cumulative survival rates (24% versus 28%, $P = 0.914$). *Conclusions*. Positive No.8p LNs were a poor but not an independent prognostic factor for patients with GC and should be recognized as distant metastasis.

1. Introduction

Gastric cancer is the second most frequent diagnosed cancer and one of the most common death-leading cancers in the world [1–4]. Gastrectomy plus appropriate lymphadenectomy is the primary treatment for resectable gastric cancer. However, there remains controversy about the degree of lymph node dissection. Two European large-scale randomized controlled clinical trials failed to prove that D2 lymphadenectomy outweighed D1 lymphadenectomy [5–8], partially due to the increased postoperative morbidity, mortality, and reoperation rate without increasing survival rate. Then, further studies revealed that D2 lymphadenectomy was also associated with lower local-regional recurrence, gastric-cancer-related death, and a better survival benefit [9–11]. However, Jiang et al. conducted a meta-analysis of 12 randomized controlled trials that showed no better overall survival benefit from D2 lymphadenectomy than that of D1 lymphadenectomy [12]. Therefore, the current consensus in West countries is gastrectomy plus D1 or modified D2 lymphadenectomy for gastric cancer [2, 13]. While clinical experience from observational or randomized controlled trails in Asia demonstrated D2 lymphadenectomy could lead to better outcomes than D1 lymphadenectomy. Thus D2 lymphadenectomy is recommended as the standard procedure for resectable gastric cancer according to the treatment guideline of Japanese Gastric Cancer Association (JGCA) [14]. As for D2 lymphadenectomy plus the extraregional lymph nodes (e.g., No.13 LNs or No.16 LNs), the results of previous studies were not cogent enough because of their own limitations [15, 16]. No.8p LNs were defined as posterior lymph nodes along the common hepatic artery and also classified as the extraregional lymph nodes [17]. It had long been theorized that prognosis of patients with evident metastatic No.8p LNs was poor, but little data was available. In this retrospective study, we aimed to analyze the prognostic value of No.8p LNs in patients with gastric cancer.

2. Method

2.1. Patients. From August 2002 to December 2011, clinicopathological and survival data of 284 GC patients who

Table 1: Details of clinicopathological characteristics and univariate correlation analysis of No.8p LNs.

	Characteristics	No.8p LNs (positive) $n = 24$ (%)	No.8p LNs (negative) $n = 260$ (%)	P value
Age	Years	54.8 ± 15.0 (27–85)	55.8 ± 11.6 (26–80)	0.685*
	>60	8 (33.3)	105 (40.4)	0.499
	≤60	16 (66.7)	155 (50.6)	
Gender	Male	16 (66.7)	168 (64.6)	0.840
	Female	8 (33.3)	92 (35.4)	
Longitudinal location	U	5 (20.8)	61 (23.5)	0.263
	M	7 (29.2)	60 (33.1)	
	L	11 (45.8)	134 (51.5)	
	Combined	1 (4.2)	5 (1.9)	
Cross-sectional location	Lesser	11 (45.8)	130 (50.0)	0.914
	Greater	4 (16.7)	30 (11.5)	
	Anterior	1 (4.2)	13 (5.0)	
	Posterior	1 (4.2)	19 (7.3)	
	Multiwalls	7 (29.2)	68 (26.2)	
Differentiation grade†	Differentiated	5 (20.8)	81 (31.2)	0.292
	Undifferentiated	19 (79.2)	179 (68.3)	
Diameter (cm)	Mean ± SD	7.06 ± 4.31	5.06 ± 3.00	0.003*
	EGC	0 (0)	8 (3.1)	0.574
Macroscopic type	Borrmann-I	1 (4.2)	10 (3.8)	
	Borrmann-II	11 (45.8)	145 (55.8)	
	Borrmann-III	8 (33.3)	75 (28.8)	
	Borrmann-IV	4 (26.7)	22 (8.5)	
Metastatic LNs	Number	18.5 ± 11.8	5.2 ± 6.4	0.000*
Harvested LNs	Number	31.0 ± 11.9	33.5 ± 12.1	0.333*
LN metastatic ratio	Percent	57.0	19.3	<0.001
Gastrectomy	Total	13	84	0.042
	Subtotal	11	176	
Curative degree	R0	16 (66.7)	237 (91.2)	0.000
	R1/R2	8 (33.3)	23 (8.8)	
Lymph node resection	D1+	10 (41.7)	66 (25.4)	0.085
	D2+	14 (58.3)	194 (74.6)	

*Student's t-test. †Histologic differentiation grade is based on the Japanese classification of gastric carcinoma: 3rd English edition
EGC: early gastric cancer.

underwent total or subtotal gastrectomy with D1+ or D2+ lymphadenectomy plus No.8p LNs dissection in Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, were retrospectively analyzed. Patients were included in this study based on the following principles: (1) preoperatively histological confirmation of gastric adenocarcinoma, (2) gastrectomy with lymphadenectomy plus No.8p LNs dissection, and (3) no remnant gastric cancer patients. Patients were divided into the No.8p-positive group (No.8p+ group) and the No.8p-negative group (No.8p− group) according to No.8p LNs status reported in postoperative pathology.

2.2. Extent of Lymphadenectomy. D1+ lymphadenectomy was regarded as gastrectomy with extended lymphadenectomy exceeding D1 but not reaching D2. While D2+ lymphadenectomy was defined as gastrectomy with lymphadenectomy beyond D2, such as lymph nodes around the area of posterior surface of the pancreatic head (No.13), superior mesenteric vein (No.14v), or the para-aortic (No.16), and so forth. The principles above were applied according to Japanese Gastric Cancer Treatment Guidelines [14]. In this study, all cases underwent D1+ or D2+ lymphadenectomy plus No.8p LNs dissection.

After the dissection of suprapyloric lymph nodes, No.8a LNs were gripped in the root of arteria gastroduodenalis. The contour of the common hepatic artery was confirmed before it was barred from its initial to the proper hepatic artery. Then No.8p LNs could be dissected in vivo by the operating surgeon. The surgery related data was recorded in the advanced database of the department.

2.3. Clinicopathological Data. The clinicopathological features contained age, gender, tumor location (longitudinal and

cross-section location), histological and macroscopic type, number of harvested and metastatic lymph nodes, and tumor stage. The histological types were categorized into differentiated type and undifferentiated type. The former consisted of well, moderate, and poor differentiated adenocarcinoma, while the latter was made up of signet-ring cell carcinoma, mucinous adenocarcinoma, papillary adenocarcinoma, and undifferentiated adenocarcinoma. Tumor staging was conducted according to the tumor-node-metastasis system of Japanese Gastric Cancer Association [17].

2.4. Follow-Up. Patients underwent regular follow-up through outpatient visit, mails, or telephones. The last follow-up was updated to December 31st, 2014. The follow-up time ranges from 36 months to 116 months. 21 cases were lost to follow-up and the lost rate was 14.4%. Overall 3-year survival (OS) rate was evaluated in this study.

2.5. Statistical Analysis. Continuous variables were presented as mean ± standard deviation and analyzed using with the Mann-Whitney U test. Categorical data was analyzed by the means of the Chi-square test or Wilcoxon test as appropriate. The risk factors of No.8p LNs metastasis were analyzed by Rank-Sum test and Chi-square test for univariate analysis and logistic regression for multivariate analysis. OS curves of patients between subgroups were calculated by Kaplan-Meier method from the day of operation to the final follow-up or death, and differences between the survival curves were assessed by log-rank test. Cox proportional hazards model was used to identify prognostic factors in univariate and multivariate analysis. The two side's P value <0.05 was considered as statistic significant. Statistical analysis was conducted by the Statistical Package for Social Science version 19.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Correlation Analysis between Clinicopathological Features and Metastasis of No.8p LNs. 24 cases of 284 patients (8.5%) showed positive metastasis of No.8p LNs. Clinicopathological features of patients were analyzed between the two groups (Table 1). Significant differences were found in tumor diameter ($P = 0.003$), mean number of metastatic lymph nodes ($P = 0.000$), types of gastrectomy ($P = 0.042$), and curative degrees ($P = 0.000$) owing to M1 disease, but no statistic differences were found in age ($P = 0.685$), gender ($P = 0.840$), tumor location ($P > 0.05$), mean number of harvested lymph nodes ($P = 0.333$), macroscopic types ($P = 0.574$), differentiation grade ($P = 0.292$), and lymphadenectomy ($P = 0.085$). Moreover, the lymph node metastatic ratio was 57.0% in the No.8p+ group and 19.3% in the No.8p− group ($P < 0.001$). Patients suffered more advanced T stages ($P = 0.024$), N stages ($P = 0.000$), and M stages ($P = 0.000$) in No.8p+ group than these of No.8p− group (Table 2).

Logistic regression verified that metastasis of No.8p LNs was closely related to positive No.8a LNs (hazard ratio [HR], 4.437; $P = 0.040$) compared with regional lymph nodes, other extraregional lymph nodes (e.g., No.13, No.15, and No.16), tumor location.

Table 2: Details of pathological stage and univariate correlation analysis of No.8p LNs.

	Characteristics	No.8p+ group $n = 24$ (%)	No.8p− group $n = 260$ (%)	P value
T stage	T1	1 (4.2)	45 (17.3)	0.024
	T2	0 (0)	36 (13.8)	
	T3	1 (4.2)	21 (8.1)	
	T4	22 (91.6)	158 (60.8)	
N stage	N0	0 (0.0)	82 (31.5)	0.000
	N1	0 (0.0)	47 (18.1)	
	N2	2 (8.3)	52 (20.0)	
	N3a	9 (37.5)	56 (21.5)	
	N3b	13 (54.2)	23 (8.8)	
M stage	M0	0 (0)	236 (90.8)	0.000
	M1*	24 (100.0)	24 (9.2)	
pTNM stage	IA	0 (0)	36 (13.8)	0.000
	IB	0 (0)	17 (6.5)	
	IIA	0 (0)	23 (8.8)	
	IIB	0 (0)	30 (11.5)	
	IIIA	0 (0)	34 (13.1)	
	IIIB	0 (0)	46 (17.7)	
	IIIC	0 (0)	48 (18.5)	
	IV	24 (100.0)	26 (10.0)	

pTNM stage is based on the Japanese classification of gastric carcinoma: 3rd English edition.
*M1 include positive extraregional lymph nodes ($n = 13$), peritoneal metastasis ($n = 9$), hepatic metastases ($n = 1$), and Krukenberg tumor ($n = 1$).

3.2. Morbidity and Mortality. No patient died within postoperative 30 days. No difference existed among operating time, intraoperative blood loss, and postoperative hospital stay between the two groups (Table 3). In the No.8p− group, the most common complications were gastroparesis (1.2%), followed by paralytic intestinal obstruction (0.8%), fistula (0.4%), abdominal hemorrhage (0.4%), and intra-abdominal infection (0.4%). Only one case of anastomosis fistula (4.1%) was found in No.8p+ group.

3.3. Survival Outcomes and Variate Analysis. Overall 3-year survival rate was 26.0% in No.8p+ group and 53.0% in No.8p− group ($P = 0.005$). We mainly explored the comparison of survival outcomes in No.8p− group at stage III/IV, because patients at stage I/II in No.8p− group did not reach their median survival time until the latest follow-up (Table 4). Significant difference of 3-year overall survival rates of the two groups existed in the items of gender, age gastrectomy, pathological degree, and curative degree ($P < 0.050$). Univariate analysis revealed that R1/R2 ($P = 0.000$), subtotal gastrectomy ($P = 0.007$), advanced T stage ($P < 0.050$),

Table 3: Comparison of morbidity and mortality between No.8p+ and No.8p− groups.

	Characteristics	No.8p+ group $n = 24$ (%)	No.8p− group $n = 260$ (%)	P value
Operating time	min	249 ± 41	260.1 ± 48.9	0.457
Intraoperative blood loss	mL	161 ± 104	168 ± 97	0.295
Postoperative hospital stay	Day	11 ± 5	11 ± 4	0.961
Overall complications	Anastomosis fistula	0	1	0.717
	Lymphatic chylous fistula	1	0	
	Paralytic intestinal obstruction	0	1	
	Abdominal hemorrhage	0	1	
	Intra-abdominal infection	0	3	
	Gastroparesis	1	14	
	Others*	0	2	

*Others include pulmonary infection ($n = 1$), delirium ($n = 1$).

Table 4: The comparison of GC patient survival outcomes in No.8p+ group and in No.8p− group at stage III/IV.

	No.8p+ group 3-year OS (%)	No.8p− group 3-year OS (%)	P value
Total	26.0	53.0	0.005
Gender			0.040
Male	27.0	51.0	
Female	25.0	56.0	
Age (years)			0.011
>60	14	40	
≤60	31	61	
Gastrectomy			
Total Gastrectomy	25	61	0.006
Subtotal gastrectomy	27	43	
Pathological degree			
Differentiated	33	55	0.041
Undifferentiated	10	47	
Curative degree			0.003
R0	19	55	
R1/R2	0	40	
Lymphadenectomy			
D1+	22	51	0.115
D2+	29	57	

pTNM stage in the No.8p− group is based on the Japanese classification of gastric carcinoma: 3rd English edition.

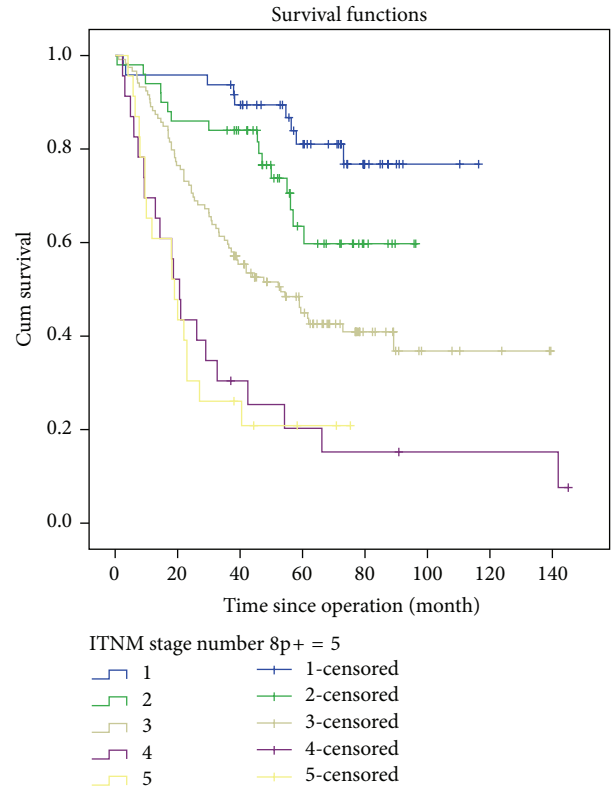

Figure 1: Cumulative survival rates categorized by tumor stage and No.8p status. No.8p-negative in stage IV versus No.8p-positive, $P = 0.923$. No.8p-negative in stage I/II/III versus No.8p-positive, $P < 0.050$. log rank test. pTNM stage is based on the Japanese classification of gastric carcinoma: 3rd English edition.

distant metastasis ($P = 0.000$), and positive No.8p LNs ($P = 0.000$) brought about higher risks of worse overall survival in GC patients, while multivariate analysis also illustrated R1/R2, T4 stage and N3b stage could run higher risks of worse overall survival in GC patients ($P < 0.050$) (Table 5). Moreover, the cumulative survival rate of No.8p− group in stage IV showed no statistical difference from that of No.8p+ group ($P = 0.923$). The cumulative survival rates of No.8p− group in stage I/II/III presented statistical difference from that of No.8p+ group ($P < 0.050$) (Figure 1). Patients in the No.8p+ group showed no statistical difference of cumulative survival rates, whether they had other extraregional lymph nodes or not ($P = 0.914$) (Figure 2).

Table 5: Univariate and multivariate analysis of prognostic factors on overall survival in patients gastric cancer based on Cox proportional hazards model.

Variables	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age (years)				
>60	1			
≤60	0.742 (0.524, 1.050)	0.092		
Gastrectomy				
Subtotal gastrectomy	1		1	
Total gastrectomy	1.778 (1.251, 2.252)	0.001	1.166 (0795, 1.710)	0.443
Tumor location				
Upper	1			
Middle	0.652 (0.384, 1.107)	0.113		
Lower	0.536 (0.363, 0.790)	0.002		
Total	2.154 (0.772, 6.010)	0.083		
Pathological degree				
Differentiated	1			
Undifferentiated	0.949 (0.653, 1.378)	0.782		
Curative degree				
R0	1		1	
R1/R2	3.926 (2.095, 7.356)	0.000	2.452 (1.267, 4.746)	0.008
Lymphadenectomy				
D1+	1			
D2+	1.215 (0.810, 1.823)	0.347		
T stage				
T1	1		1	
T2	3.257 (1.147, 9.246)	0.027	2.514 (0.856, 7.387)	0.094
T3	3.697 (1.170, 11.687)	0.026	2.724 (0.833, 8.911)	0.097
T4	7.791 (3.173, 19.128)	0.000	4.556 (1.725, 12.035)	0.002
N stage				
N0	1		1	
N1	1.513 (0.798, 2.870)	0.205	1.024 (0.527, 1.990)	0.943
N2	1.935 (1.082, 3.461)	0.026	1.124 (0.605, 2.088)	0.711
N3a	3.429 (2.110, 7.103)	0.000	1.771 (0.986, 3.182)	0.056
N3b	7.245 (4.075, 12.880)	0.000	3.644 (1.952, 6.801)	0.000
M stage				
M0	1		1	
M1	3.223 (2.137, 4.861)	0.000	1.551 (0.877, 2.744)	0.132
No.8p LNs status				
Negative	1		1	
Positive	3.101 (1.873, 5.136)	0.000	0.892 (0.469, 1.696)	0.728

Backward variable selection with selection criteria of 0.2 was conducted with all clinicopathologic variables. pTNM stage is based on the Japanese classification of gastric carcinoma: 3rd English edition.

4. Discussion

Extent of lymph node dissection has been a decade-old argument, since D2 lymphadenectomy was recommended to be performed by experienced surgeons in West and the standard procedure in Japan [5–14]. Some of the extraregional lymph nodes had been reported [15, 16], but the prognostic value of No.8p LNs dissection is still unclear. Our study showed that positive No.8p LNs should be defined as distant metastasis rather than regional lymph node metastasis and positive No.8p LNs was a poor prognostic factor for GC patients.

Maruyama et al. reported that the incidence of metastasis of No.8 LNs was about 16% [18], and Sasako et al. reported that the therapeutic index of No.8 LNs was 5.9 by multiplying the metastatic frequency of No.8 LNs with the 5-year survival rate of patients with positive No.8 LNs [19]. The two studies focus mainly on No.8a LNs because of fewer metastasis of No.8p LNs. Based on this kind of therapeutic index and lymphatic flow at different tumor sites, No.8p LNs were recognized

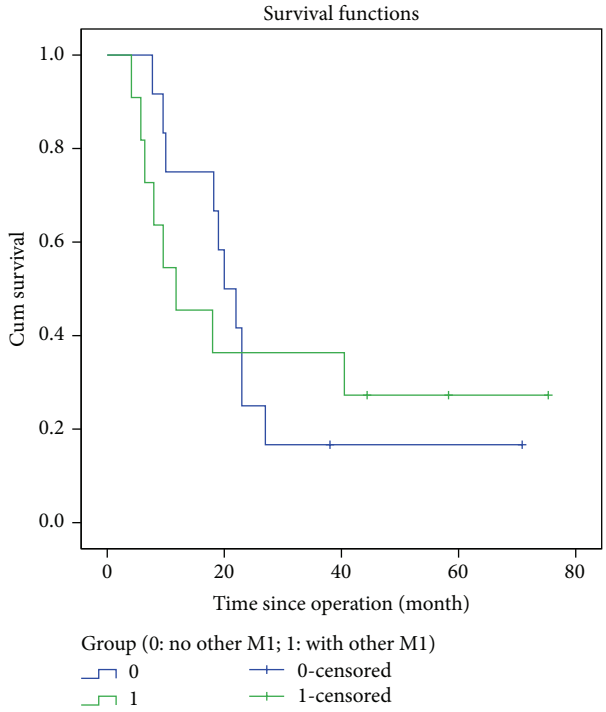

Figure 2: Cumulative survival rates categorized by distant metastasis in No.8p-positive group, $P = 0.914$, No.8p-positive group without other M1 versus No.8p-positive group with other M1. log rank test. pTNM stage is based on the Japanese classification of gastric carcinoma: 3rd English edition.

as the extraregional lymph nodes [17]. In this study, we confirmed that metastasis rate of No.8p LNs was 8.5%, which was consistent with previous reports [18, 19]. But we failed to calculate the therapeutic index of No.8p LNs as Sasako et al. did, mainly because of the short follow-up time and relative small sample size. Hence, more high-quality and large-sample trials and long-term follow-up are necessary to assess the therapeutic index of No.8p LNs. Another characteristic, we need to notice, was the metastasis of No.8p LNs that was closely related to that of No.8a LNs compared with the other lymph nodes, tumor location. This might be in accordance with their anatomical relationship and lymphatic flow.

Patients in the No.8p+ group suffered more advanced pathological stage. Moreover, patients in the No.8p− group enjoyed better survival in the I/II/III stage than those in the No.8p+ group ($P < 0.050$), but patients in the No.8p− group showed no survival difference in the IV stages compared with patients in the No.8p+ group ($P = 0.923$). Even after R0 resection, the 3-year survival rate in No.8p+ group was much lower than that in No.8p− group ($P = 0.003$). Moreover, lymph node metastatic ratio had been advocated to be a more appropriate method for N stage and predicted survival partly in recent years [20, 21]. From our study, patients in the No.8p+ group had a higher lymph node metastatic ratio than that in the No.8p− negative group ($P < 0.001$). At pN3 stage, the lymph node metastatic ratio in the No.8p+ group was 0.446 at pN3a stage and 0.740 at pN3b stage, respectively. We also demonstrated that the 3-year survival rate in the No.8p+ group was 50.0% at pN3a stage and 8.0% at pN3b stage, respectively. Moreover, univariate analysis revealed positive No.8p LNs ($P = 0.000$) brought about higher risks of worse overall survival, while multivariate analysis illustrated that positive No.8p LNs was not an independent prognostic factor in GC patients. All the factors above demonstrated that No.8p LNs were extraregional lymph nodes rather than regional lymph nodes and that metastasis of No.8p LNs should be recommended as distant metastasis, which was in accordance with the Japanese Classification of Gastric Carcinoma [17].

5. Conclusion

It was acknowledged that the number of cases was small in No.8p+ group. Higher rate of other M1 diseases made R0 rate less in No.8p+ group. However, survival outcomes between cases in No.8p+ group and IV stage cases in No.8p− group indicated that positive No.8p LNs was a poor but not an independent prognostic factor for patients with gastric cancer and should be recognized as the distant metastasis rather than regional lymph node metastasis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank the substantial work of the Volunteer Team of Gastric Cancer Surgery (VOLTGA), West China Hospital, Sichuan University, China. The domestic support was received from (1) National Natural Science Foundation of China (no. 81372344, 81301867); (2) New Century Excellent Talents in University support program, Ministry of Education of China (2012SCU-NCET-11-0343).

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 326581, 12 pages
http://dx.doi.org/10.1155/2015/326581

Clinical Study

A Randomized Controlled Trial Evaluating a Low-Volume PEG Solution Plus Ascorbic Acid versus Standard PEG Solution in Bowel Preparation for Colonoscopy

Masahiro Tajika,[1] Tsutomu Tanaka,[1] Makoto Ishihara,[1] Nobumasa Mizuno,[2] Kazuo Hara,[2] Susumu Hijioka,[2] Hiroshi Imaoka,[2] Takamitsu Sato,[2] Tatsuji Yogi,[2] Hideharu Tsutsumi,[2] Toshihisa Fujiyoshi,[2] Nobuhiro Hieda,[2] Nozomi Okuno,[2] Tsukasa Yoshida,[2] Vikram Bhatia,[3] Yasushi Yatabe,[4] Kenji Yamao,[2] and Yasumasa Niwa[1]

[1]*Department of Endoscopy, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan*
[2]*Department of Gastroenterology, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan*
[3]*Department of Medical Hepatology, Institute of Liver and Biliary Sciences, D-1, Vasant Kunj, New Delhi, Delhi 110070, India*
[4]*Department of Pathology and Molecular Diagnosis, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan*

Correspondence should be addressed to Masahiro Tajika; mtajika@aichi-cc.jp

Received 9 February 2015; Accepted 2 April 2015

Academic Editor: Miroslav Zavoral

Evaluation of polyethylene glycol electrolyte lavage solution containing ascorbic acid (PEG-ASC) has been controversial in the point of its hyperosmolarity, especially in old population. So we therefore designed the present study to compare the efficacy, acceptability, tolerability, and safety of 1.5 L PEG+ASC and 2 L standard PEG electrolyte solution (PEG-ELS), not only in the general population, but also in patients of advanced age. Randomization was stratified by age (<70 years or 70> years), and hematological and biochemical parameters were compared in each age group, especially with respect to the safety profile of each regimen. As a result, the 1.5-L PEG-ASC regimen had higher patient acceptability than the 2-L PEG-ELS regimen. Tolerability, bowel cleansing, and safety were similar between regimens. However, we demonstrated significant statistical changes in the hematological and biochemical parameters after taking bowel preparation solutions, not only in the PEG+ASC group, but also in the PEG-ELS group. No significant differences in the safety profile were found between subjects aged less than 70 years and those aged 70 years or more; nevertheless, regardless of age, proper hydration is needed throughout the bowel preparation process.

1. Introduction

Polyethylene glycol electrolyte lavage solution (PEG-ELS) is the gold standard for bowel cleansing (strong recommendation, high quality) for colonoscopy by recommendation of the US Multi-Society Task Force on Colorectal Cancer [1] (American College of Gastroenterology, American Gastroenterological Association, and American Society of Gastrointestinal Endoscopy). However, although osmotically balanced electrolyte lavage solutions offer safe and effective cleansing [2–4], volume-related discomfort and adverse experiences have decreased the percentage of patients completing the preexamination preparation. This is mainly due to large volumes of fluid required for bowel preparation, unpleasant taste, and an increase in the incidence of side effects [3]. Although 3-4 L of PEG-ELS is used in Western countries, approximately 2 L of PEG-ELS, along with a laxative, is usually considered adequate for bowel preparation in Japan. In our hospital, standard regimen of colonoscopy preparation is a low-residue diet for one day before colonoscopy and 2 L of PEG-ELS with a laxative. Despite the lower-volume in Japan, the need to drink such large volumes of liquid with an unpalatable taste has a negative impact on patient compliance [5]. Therefore, more effective bowel preparation regimens for colonoscopy are needed to improve the acceptability

and tolerability of the procedure. In a previous randomized trial, we found that coingestion of 15 mg of Mosapride as a prokinetic was not effective for reducing the volume of PEG-ELS required for bowel preparation [6]. Nevertheless, we found that the 1.5 L PEG-ELS regimen had better patient acceptability and tolerability compared with the 2 L PEG-ELS regimen. Thus the reduction of 0.5 L PEG-ELS volume for bowel preparation is a worthwhile goal, from the patient's perspective.

Recently, a new low-volume hyperosmolar PEG-ELS has become available in Japan. It combines PEG-ELS with a high dose of ascorbic acid (PEG+ASC, Moviprep, Ajinomoto Pharmaceuticals Co., Ltd., Tokyo, Japan). The addition of ascorbic acid reduces the volume of the lavage solution from 4 L to 2 L. The cathartic effects of ascorbic acid are thought to be due to its absorption mechanism that saturates at a high dose [7, 8]. Excess ascorbic acid that cannot be absorbed remains in the bowel where it exerts an osmotic effect, acting synergistically with PEG-ELS. The addition of ascorbic acid also appears to improve the taste of the PEG-ELS preparation. In Western countries, PEG+ASC has been available for bowel preparation for colonoscopy since 2006; and several studies have demonstrated the efficacy, acceptability, tolerability, and safety of PEG+ASC compared with several regimens of standard PEG-ELS [9–20]. In Japan, phase III trial was performed that evaluates the efficacy, acceptability, tolerability, and safety of PEG+ASC compared with standard PEG-ELS without food restriction and laxative. In that study, it took 1.63 ± 0.38 L PEG+ASC to obtain the optimal colonoscopy preparation (not published data). From this result, we suppose that 1.5 L PEG+ASC with food restriction and laxative are equivalent to standard regimen in our hospital.

Because of an ageing population, and increasing burden of colorectal cancer at an advanced age, colonoscopies are increasingly needed in the elderly. Although the efficacy and safety of PEG-ELS have been proven in elderly patients, and those with comorbidities, PEG-ASC has not been evaluated in Japanese patients, particularly those with advanced age.

We therefore designed the present study to compare the efficacy, acceptability, tolerability, and safety of 1.5 L PEG+ASC and 2 L PEG-ELS, not only in the general population, but also in patients of advanced age.

2. Patients and Methods

This was a prospective, randomized, controlled, single-center, investigator-blinded, noninferiority study comparing 1.5 L PEG+ASC with 2 L PEG-ELS in patients who underwent colonoscopy. All patients provided written, informed consent. The study was conducted at Aichi Cancer Center Hospital (ACCH), Nagoya, from September 2013 to August 2014. The study was reviewed and approved by the ethics committee of ACCH, and all patients signed an approved informed consent form prior to entering the study. This trial was registered in an international clinical trial registry (UMIN000011505). All consecutive inpatients of both sexes aged 20 years and older who were scheduled for mainly therapeutic colonoscopy such as polypectomy, endoscopic mucosal resection, and endoscopic submucosal dissection at ACCH were evaluated for inclusion in the study. Patients with the following clinical features were excluded: significant cardiac, renal, hepatic, or metabolic comorbidities, ascites, severe constipation (<2 bowel movements per week), known allergy to PEG-ELS, history of gastric stapling or bypass procedure, or history of prior colonic or rectal surgery. Patients were excluded if there was a suspected diagnosis of intestinal obstruction because of advanced colorectal cancer.

2.1. Randomization and Blinding. Patients were randomly allocated to receive one of two different bowel preparation regimens using a computer-generated random-number list. Randomization was stratified by age (<70 years or 70≤ years) and performed in blocks of 4. Concealed allocation was accomplished through nonresearch personnel who were not involved in this study. Patients were instructed not to discuss their bowel preparation with anyone other than the unblinded research assistant. With the exceptions of the patient and the unblinded research assistant, all other individuals participating in this study, including the endoscopists and endoscopy nurses, were blinded to the allocated treatment group. Comparisons between the 1.5 L PEG+ASC group and the 2 L PEG-ELS group were made in an investigator-blinded fashion.

2.2. Bowel Preparation Methods (Figure 1). The day before colonoscopy, all patients were admitted to our hospital. An orientation talk was given by a nurse, who carefully explained how the product should be taken, emphasizing the importance of complete intake of the solution to ensure a safe and effective procedure. The subjects were instructed to eat a low-residue diet served in our hospital and asked to drink more than 2 liters of clear liquid. On the evening (up to 22:00) before the day of the colonoscopy, all patients were instructed to take 7.5 mg sodium picosulfate hydrate (Laxoberon: Teijin Pharma, Ltd., Tokyo, Japan). On the day of the colonoscopy, participants received either PEG+ASC (Moviprep: each liter contained 100.0 g macrogol 4000, 7.5 g sodium sulfate, 2.7 g sodium chloride, 1.0 g potassium chloride, 4.7 g ascorbic acid, 5.9 g sodium ascorbate, and lemon flavoring) or PEG-ELS (Niflec: Ajinomoto Pharmaceuticals Co., Ltd., Tokyo, Japan, each liter containing 59.0 g macrogol 4000, 5.7 g sodium sulfate, 1.5 g sodium chloride, 0.7 g potassium chloride, 1.7 g sodium bicarbonate, and lemon flavoring). The PEG-ELS group was instructed to begin drinking 2 L at a rate of 0.25 L every 15 min. The PEG+ASC group was instructed to begin drinking the first 1 L of cleansing solution followed by 0.5 L clear fluid; after that, they were instructed to begin drinking the remaining 0.5 L of cleansing solution followed by 0.25 L clear fluid at a rate of 0.25 L every 15 min. These instructions were in accordance with those of the manufacturer. All patients were instructed to take clear liquids after they finished drinking the cleansing solution. Colonoscopies were scheduled to be performed after 16:00.

2.3. Evaluation of Bowel Preparation. The efficacy of the bowel preparation was assessed using the Boston Bowel

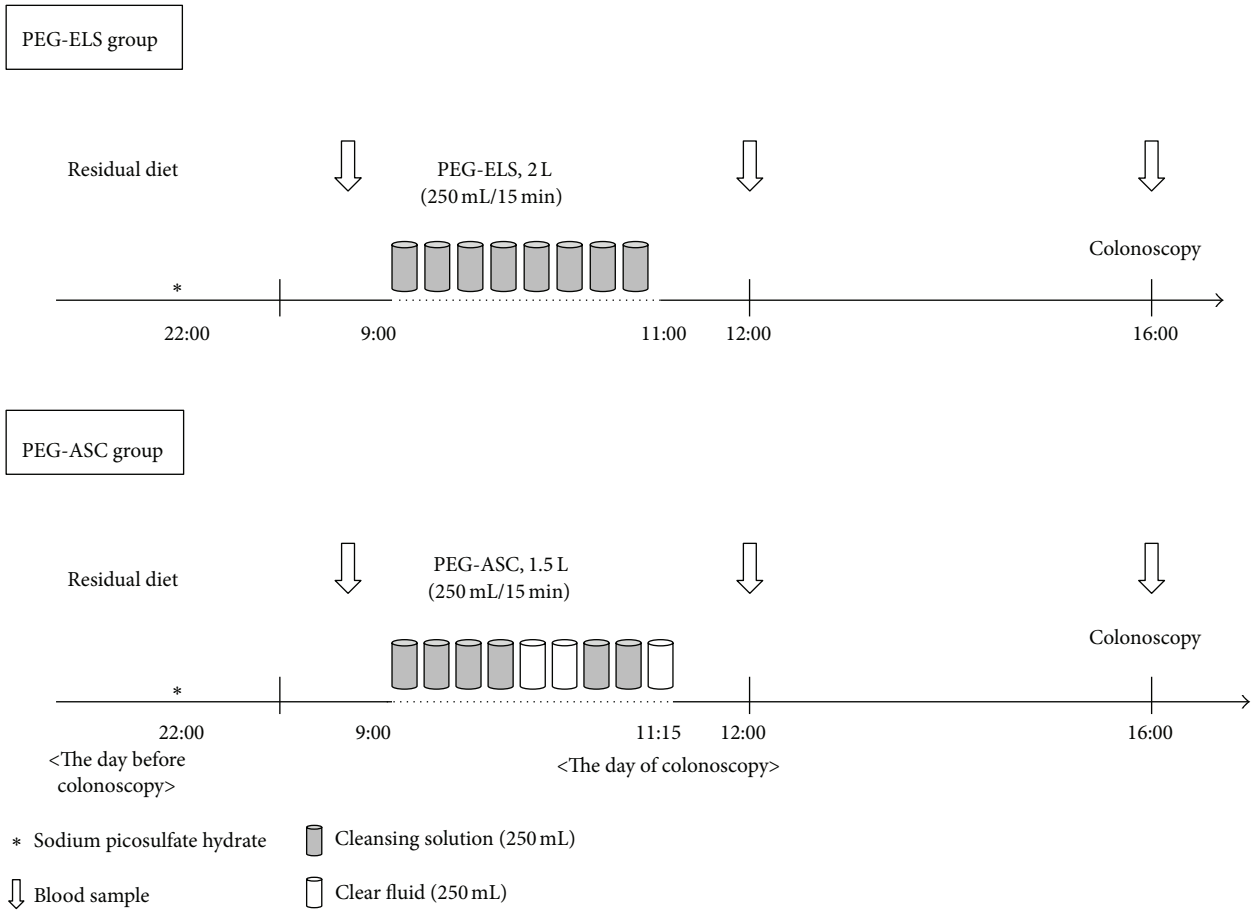

Figure 1: The study schedule. PEG-ELS, polyethylene glycol electrolyte lavage solution. PEG-ASC, polyethylene glycol electrolyte lavage solution containing ascorbic acid.

Preparation Scale (BBPS) [21]. The preparation efficacy was evaluated by the blinded endoscopist per colonic segment (right, transverse, and left colon) on a 4-point scale (0–3) according to the BBPS. In addition, overall cleansing of the colon was scored by summing up the scores of each segment. For the study, the total score of each patient, ranging from 0 to 9, was divided into four different classes: excellent cleansing (total score 8-9), good cleansing (6-7), poor cleansing (3–5), and inadequate cleansing (i.e., requiring additional treatment, 0–2). The participating endoscopists were trained to use the BBPS scale to achieve a good level of agreement. The final assessment of the bowel preparation was divided into two categories, successful and failed. A bowel preparation rated as excellent or good based on the BBPS was considered successful, and poor or inadequate ratings were considered failed. The investigators performed calibration exercises involving more than 20 colonoscopies prior to study commencement, based on their interpretation of scale anchors, to ensure that their findings agreed.

The physicians were also asked to score the overall mucosal visibility according to the following 3-grade scale [22]: optimal (grade 0, clear imaging with no or a minimal amount of bubbles or foam that could be easily removed), adequate (grade 1, modest amount of bubbles and foam that could be cleared with a minimal amount of time), and insufficient (grade 2, presence of foam and bubbles that significantly reduced the clear visualization of the mucosa).

During or immediately following the colonoscopy, the investigator completed a physician questionnaire regarding assessment of the bowel preparation, amount of irrigation fluid used, time needed to reach the cecum, ease of insertion into the cecum, and difficulty in observing the lumen of the colorectum because of peristalsis.

2.4. Patient Acceptability, Tolerability, and Other Measurements. The nursing staff recorded the time required to drink the indicated volume of lavage solution. They also recorded the time and number of bowel movements from the start of ingestion to the appearance of clear excretion. Until one hour after finishing the preparation procedure, the nursing staff checked excretions. If there was a solid stool with muddy excretions or no excretion at that time, the patient was given an additional preparation, such as additional PEG-electrolyte solution or enemas. The patients who received an additional preparation were defined by the BBPS as inadequate. The patient questionnaire consisted of 20 questions. Tolerability

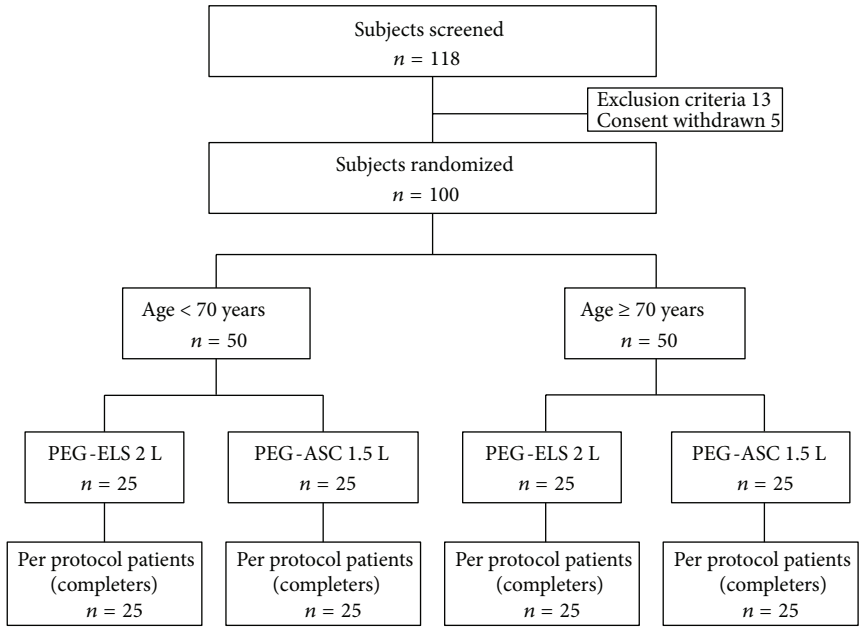

Figure 2: Patient flow.

assessment was based on the recording of GI symptoms such as nausea, vomiting, bloating, and abdominal pain. These events were scored on a 4-point scale: 1 = none, 2 = mild, 3 = moderate, and 4 = severe. The acceptability assessment was based on the willingness to repeat the same preparation regimen. The patients made an entry in the questionnaire form before undergoing colonoscopy and submitted it to the nursing staff.

Blood was sampled at baseline, and 3 hours and 7 hours after the beginning of bowel cleansing for hematological and biochemical analysis. After blood sampling at 7 hours, the scheduled colonoscopy was performed.

2.5. End Points. The primary end point was patient acceptability, defined as the rating of "much" willingness to repeat the same preparation regimen. Secondary end points included tolerability, overall colon cleansing (defined as the rate of "successful" cleansing), the rate of optimal mucosa visibility (score 0), and total time for colonic preparation.

2.6. Statistical Analysis. Based on a previous study [6], the rate of "much" willingness to repeat the same preparation regimen for the PEG-ELS was expected to be 61.9%. For an adequate rate, we expected about 75% of the PEG+ASC group to give a rating of "much" and the noninferiority margin was set at −10%. This study was designed to have 80% power to establish noninferiority (using a one-sided significance level of 0.025 and a target sample size of 100). If the difference between treatments was above this cutoff but also above the zero difference line, we defined 1.5 L PEG+ASC as superior to 2 L PEG-ELS. The primary analysis for noninferiority was performed on the per protocol (PP) population.

Baseline characteristics were summarized by the usual descriptive statistics, such as the mean and standard deviation for continuous variables and rates for categorical variables. The two-sided t-test was used to compare the mean of continuous variables; the likelihood ratio Chi-squared test was used to compare the categorical measures. The paired t-test was used to compare the mean of blood data before and after the procedure. P values less than 0.05 were considered as statistically significant.

3. Results

3.1. Patient Characteristics. A total of 100 patients were randomized into two groups (Figure 2). The baseline characteristics of the patients are shown in Table 1. There were no significant differences in age, sex, body mass index, or indications for colonoscopy between the two groups. Ninety-eight of 100 study patients had undergone colonoscopy in our hospital as outpatients in the previous 6 months, at which time colon polyps and/or early colorectal cancer were detected. Therefore, most of the study patients had already experienced bowel preparation with PEG-ELS (Niflec) and colonoscopy in our hospital previously.

3.2. Patient Acceptability, Tolerability, and Safety. Patient tolerance and acceptance, as assessed by a questionnaire scoring subjective evaluations, are shown in Table 2. There was no significant difference in compliance between the two groups, as defined by >75% intake of the prescribed bowel cleansing solution volume and complete (100%) intake of the bowel cleansing solution between the two regimens. When asked about their overall impression, the proportion of patients who answered "easy" or "difficult" was significantly different between the two groups ($P = 0.008$). However, symptoms such as nausea, vomiting, bloating, abdominal pain, and circulatory reactions were similar between groups.

Table 1: Patient characteristics.

Variable	PEG-ELS	PEG-ASC	*P* value
Number of patients	50	50	
<70/≥70	25/25	25/25	1*
Age (years)			
mean ± SD	67.9 ± 9.4	64.6 ± 13.4	0.150**
median (range)	69.5 (35–83)	68.5 (27–89)	0.841*
<70; mean ± SD	57.4 + 9.5	56.9 + 10.3	0.842**
≥70; mean ± SD	74.8 + 3.6	75.5 + 5.0	0.559**
Male/female	32/18	33/17	0.834*
<70	17/8	17/8	1*
≥70	15/10	16/9	0.770*
Body mass index (kg/m^2)	22.8 + 3.8	22.8 + 4.1	0.982**
Reason for colonoscopy, *n*			
EMR/ESD	49	50	1*
Anemia and advanced age	1	0	

*Chi-square test; **Welch's test. EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection.

The primary end point of this study, the rate of patients who declared that they would be willing to repeat the same preparation regimen if needed, was significantly higher in the 1.5 L PEG+ASC group (72%) compared with the 2 L PEG-ELS group (52%). This gave a difference of +20% with a lower limit of the 1-sided 97.5% confidence limit of 1.4% (i.e., within the limits for noninferiority and more superiority set before the study). Furthermore, among the subgroup of 1.5 L PEG+ASC group, who had undergone bowel preparation with 2 L PEG-ELS for colonoscopy within the previous 6 months, 24 of 50 (48%) patients felt that it was easier to ingest 1.5 L PEG+ASC than 2 L PEG-ELS.

3.3. Bowel Cleansing Efficacy. The efficacy of bowel preparation is shown in Table 3. There was no significant difference in the successful bowel preparation rate between the 1.5 L PEG+ASC group (92%) and the 2 L PEG-ELS group (82%). The mucosal inspection was rated optimal in 56.4% of the 2 L PEG-ELS group and in 48.0% of the 1.5 L PEG+ASC group ($P = 0.159$). Two patients (4.0%) in each group required additional preparation.

The time to first defecation and the completion time for bowel preparation were significantly shorter in the 1.5 L PEG+ASC group than in the 2 L PEG-ELS group ($P = 0.041$, $P = 0.030$; resp.). There were no differences between the two groups in frequency of defecation, elapsed time from last fluid intake to colonoscopy, amount of irrigation fluid used, time needed for endoscopist to reach the cecum, and subjective difficulties in insertion to the cecum.

3.4. Hematological and Biochemical Measurements. Table 4 shows the mean change from screening to 3 hours and to 7 hours after the baseline in both groups. Although many significant fluctuations in hematological and biochemical parameters were noted within both groups, all parameters except sodium bicarbonate at 7 hours after beginning the regimen in the 1.5 L PEG+ASC group changed within the normal range. In the PEG-ELS group, serum albumin, alanine aminotransferase (ALT), and pH were significantly increased at both 3 hours and 7 hours. Serum glucose, sodium, chloride, phosphorus, magnesium, and osmolarity were significantly decreased at both 3 hours and 7 hours. In the 1.5 L PEG+ASC group, serum total protein, albumin, ALT, red blood cells, and hematocrit were significantly increased at both 3 hours and 7 hours. Serum sodium, chloride, pH, sodium bicarbonate, base excess, and osmolarity were significantly decreased at both 3 hours and 7 hours.

Comparison of hematological and biochemical changes in patients under 70 years of age is shown in Table 5. Regarding parameter differences between 3 hours and the beginning, the differences in the serum creatinine, chloride, and magnesium values were significantly larger in the 2 L PEG-ELS group, and the differences in the serum phosphorus, pH, sodium bicarbonate, and base excess values were significantly larger in the 1.5 L PEG+ASC group. Regarding the differences between 7 hours and the beginning, the differences in the blood urea nitrogen (BUN), serum potassium, chloride, and phosphorus values were significantly larger in the 2 L PEG-ELS group, and the differences in the pH, sodium bicarbonate, base excess, red blood cell, and hematocrit values were significantly larger in the 1.5 L PEG+ASC group.

Comparison of hematological and biochemical changes in patients aged 70 years and over is shown in Table 6. Regarding the differences between 3 hours and the beginning, the differences in the serum chloride and magnesium values were significantly larger in the 2 L PEG-ELS group, and the differences in the serum creatinine, phosphorus, pH, sodium bicarbonate, and base excess values were significantly larger in the 1.5 L PEG+ASC group. Regarding the differences between 7 hours and the beginning, the differences in the chloride and phosphorus values were significantly larger in

Table 2: Patient tolerance and acceptance.

Variable	PEG-ELS	PEG-ASC	*P* value
Number of patients	50	50	
Compliance >75% (*n*, %)	45 (90)	47 (94)	0.461*
100% intake (*n*, %)	44 (88)	44 (88)	1*
How was the taste of preparation liquid?			
(very good/good/fair/bad/unacceptable)	(0/3/28/17/2)	(0/9/27/13/1)	0.099**
At what volume did you feel distress?			
(<500/<1000/<1500/<2000/no distress)	(3/12/21/5/9)	(6/15/15/0/14)	0.537**
How easy/difficult to take preparation (overall impression)?			
(very easy/easy/fair/difficult/very difficult)	(1/4/24/15/6)	(1/16/20/11/2)	**0.008****
Any symptoms (*n*)			
Nausea			
(none/mild/moderate/severe)	(42/4/2/2)	(41/6/3/0)	0.870**
Vomiting			
(no/yes)	(48/2)	(49/1)	0.558*
Distension			
(none/mild/moderate/severe)	(8/22/18/2)	(16/21/9/4)	0.079**
Abdominal pain			
(none/mild/moderate/severe)	(43/5/2/0)	(43/6/1/0)	0.973**
Circulatory reactions			
(none/mild/moderate/severe)	0	0	1**
Willingness to repeat (*n*) the same preparation regimen			
(much/somewhat/never)	(26/9/15)	(36/7/7)	**0.031****
Willingness to repeat (rate of much, %)	52	72	**0.039**
Point estimate and 95% CI the difference between for willingness to repeat	**+20 (0.014, 0.386)**		
How easy/difficult to undergo this preparation compared with previous one, PEG (*n*)			
(easy/intermediate/difficult)		(24/19/7)	

PEG, polyethylene glycol; ASC, ascorbic acid; N.S., not significant.
*Chi-square test, **Mann-Whitney *U* test.

Table 3: Results of the preparation and endoscopic findings.

Variable	PEG-ELS	PEG-ASC	*P* value
Number of patients	50	50	
Time to first defecation (min, mean ± SD)	64 ± 41	49 ± 33	**0.041***
Frequency of defecation (times, median, quartile)	8 (3–31)	7 (3–15)	0.160**
Time to preparation (min, mean ± SD)	194 ± 69	172 ± 77	**0.030***
Elapsed time from last fluid intake to colonoscopy (min, mean ± SD)	217 ± 80	238 ± 81	0.181*
Cecal intubation rate (*n*, %)	50 (100)	50 (100)	1.000***
Insertion time (min, median, quartile)*	7.5 (3–30)	7 (3–21)	0.689*
Feel of peristalsis (*n*, %)	9 (18)	10 (20)	0.799***
Qualitative preparation rating (*n*, %)			
Excellent	24	22	
Good	17	24	0.889**
Poor	7	2	
Inadequate (additional treatment)	2	2	
Successful bowel cleansing (*n*, %)	41 (82)	46 (92)	0.234***
Optimum visibility grade (0/1/2)	31/14/5	24/17/9	0.133**
Amount of irrigation fluid (*n*)			
None	10	8	
≤100 mL	32	24	0.063**
100 mL<	8	18	

*Welch test; **Mann-Whitney *U* test; ***Chi-square test.

Table 4: Laboratory results comparing baseline to 3 hours and 7 hours after taking the gut lavage solution.

		PEG-ELS			*P* value		PEG-ASC			*P* value	
		I	II	III	I vs II	I vs III	I	II	III	I vs II	I vs III
Total protein (g/dL)	6.7–8.3	6.9	7.0	7.0	0.086	0.223	6.9	7.2	7.1	**<0.001**	**<0.001**
Albumin (g/dL)	4.0–5.0	4.2	4.3	4.3	0.015	0.004	4.2	4.4	4.4	**<0.001**	**<0.001**
Glucose (mg/dL)	70–109	100.0	92.2	92.0	0.002	<0.001	93.1	89.5	90.6	0.076	0.320
BUN (mg/dL)	8–22	13.5	14.7	12.8	0.513	<0.001	13.3	13.3	13.5	0.917	0.441
Creatinine (mg/dL)	0.60–1.10	0.74	0.72	0.73	0.077	0.451	0.74	0.76	0.74	**0.003**	0.344
ALT (U/L)	6–30	23.4	26.0	25.4	**<0.001**	**<0.001**	23.4	26.7	26.9	**<0.001**	**<0.001**
Sodium (mmol/L)	138–146	140.4	139.7	139.5	**0.015**	**0.006**	140.5	139.9	139.1	**0.033**	**<0.001**
Potassium (mmol/L)	3.6–4.9	4.2	4.1	3.9	0.264	**<0.001**	4.2	4.3	4.1	**0.009**	**0.004**
Chloride (mmol/L)	99–109	105.1	103.5	102.9	**<0.001**	**<0.001**	105.1	105.9	104.9	**0.003**	0.422
Calcium (mg/dL)	8.7–10.3	9.1	9.0	9.0	0.148	0.189	9.1	9.2	9.2	0.082	0.665
Phosphorus (mg/dL)	2.5–4.7	3.5	3.5	3.3	**0.086**	**<0.001**	3.5	3.8	3.4	**<0.001**	0.752
pH	7.32–7.42	7.38	7.39	7.39	**0.001**	**0.005**	7.38	7.35	7.35	**<0.001**	**<0.001**
Sodium bicarbonate	24.0–28.0	27.5	27.4	26.8	0.802	0.062	28.1	24.2	23.8	**<0.001**	**<0.001**
Base excess (mmol/L)	−2.5–2.5	1.6	1.8	1.4	0.331	0.415	2.1	−1.7	−2.0	**<0.001**	**<0.001**
Osmolality (mOsm/kg)	285–295	291.2	289.8	288.6	**0.059**	**<0.001**	290.9	289.6	288.1	**0.011**	**<0.001**
Magnesium (mg/dL)	1.8–2.4	2.1	2.0	2.0	**<0.001**	**<0.001**	2.0	2.1	2.0	0.141	**<0.001**
White blood cells (/μL)	3400–8400	5622.8	5853.6	6732.8	0.129	**<0.001**	5753.2	5807.4	7473.4	0.636	**<0.001**
Red blood cells ($\times 10^4/\mu$L)	420–560	431.2	424.5	430.3	0.306	0.729	446.3	455.7	454.2	**0.001**	**0.002**
Hemoglobin (g/dL)	M: 13.5–18.0 F: 11.3–14.9	13.6	13.5	13.5	0.363	0.171	13.9	14.0	14.6	0.912	0.261
Hematocrit (%)	M: 39.9–50.3 F: 34.8–44.0	40.9	40.7	40.6	0.500	0.304	41.6	42.7	42.5	**0.001**	**0.005**
Neutrophils (%)		58.3	60.1	59.0	**0.039**	0.522	59.0	62.8	63.4	**0.001**	**<0.001**
Lymphocytes (%)		30.7	29.7	31.6	0.142	0.236	30.7	27.9	28.5	**0.005**	**0.032**
Eosinophils (%)		2.7	2.1	1.8	**<0.001**	**<0.001**	2.8	2.1	1.7	**<0.001**	**<0.001**

I, before preparation; II, 3 hrs after beginning of preparation; III, 7 hrs after beginning of preparation; *P* value by paired *t*-test two-side. BUN, blood urea nitrogen; ALT, alanine aminotransferase.

the 2 L PEG-ELS group, and the differences in the pH, sodium bicarbonate, and base excess values were significantly larger in the 1.5 L PEG+ASC group.

There were no statistically or clinically significant differences between subjects under or over 70 years of age in hematological and biochemical changes between before and after taking 2 L PEG-ELS or 1.5 L PEG+ASC.

4. Discussion

Colonoscopy is considered the most effective procedure for early detection and prevention of colorectal cancer [23, 24]. Adequate bowel preparation is essential for an effective colonoscopy evaluation [1]. Inadequate bowel preparation can result in missed lesions, aborted procedures, and increased discomfort as well as a potential increase in complication rates [25–28]. Large-volume PEG-ELS preparations are used mainly because of their favorable safety profile and proven efficacy so far [2, 3]. However, the major limitation of their use is the volume of preparation to be ingested, which may have a negative impact on patient acceptability, compliance, and, as a result, reduction in overall efficacy [26, 27].

Several low-volume regimens have been introduced into clinical practice. They are based on the combination of low-volume PEG-ELS with a stimulant laxative—senna or bisacodyl [29, 30] or a prokinetic—mosapride or itopride [31, 32]. These low-volume regimens have shown similar efficacy and higher acceptability than the standard dose of PEG-ELS [29–32]. Current trends are leading towards an increase in the use of low-volume preparations, and the combination of 2 L PEG+ASC is being considered as a market leader in the Western countries. Recently, PEG+ASC has become available in Japan. Although the efficacy, acceptability, tolerability, and safety of PEG+ASC compared with standard-volume PEG-ELS have already been demonstrated in the Western countries, PEG+ASC has never been evaluated in Japanese patients.

The present study demonstrated that 1.5 L PEG+ASC solution was not inferior, but instead superior to 2 L PEG-ELS in patient acceptability of bowel preparation for colonoscopy. At the same time, 1.5 L PEG+ASC was similar to 2 L PEG-ELS in bowel cleansing efficacy, tolerability, and safety. These results are in line with previous studies [9–20] that compared the efficacy, acceptability, tolerability, and safety of low-volume PEG+ASC and standard-volume PEG-ELS for bowel preparation for colonoscopy. Ell et al. [9] showed

Table 5: Differences in laboratory results between baseline and 3 hours and 7 hours after taking gut lavage solution in patients aged less than 70 years.

	Difference between 3 hrs and baseline			Difference between 7 hrs and baseline		
	PEG-ELS	PEG-ASC	*P*-value	PEG-ELS	PEG-ASC	*P*-value
Total protein (g/dL)	0.1 (0.4)	0.4 (0.4)	0.013	0.0 (0.4)	0.2 (0.3)	0.068
Albumin (g/dL)	0.1 (0.2)	0.3 (0.3)	0.044	0.1 (0.3)	0.2 (0.2)	0.152
Glucose (mg/dL)	−5.2 (9.7)	−2.9 (6.8)	0.325	−11.1 (19.6)	−6.4 (8.1)	0.279
BUN (mg/dL)	−0.6 (1.2)	0.1 (1.5)	0.072	−0.9 (1.3)	0.0 (1.5)	**0.023**
Creatinine (mg/dL)	−0.02 (0.05)	0.01 (0.05)	**0.030**	−0.02 (0.05)	0.02 (0.05)	**0.025**
ALT (U/L)	2.5 (2.6)	3.4 (2.9)	0.218	2.2 (2.9)	3.3 (3.4)	0.202
Sodium (mmol/L)	−0.8 (2.4)	−0.7 (2.2)	0.903	−1.2 (2.7)	−1.3 (1.6)	0.901
Potassium (mmol/L)	0.0 (0.5)	0.1 (0.2)	0.117	−0.3 (0.3)	−0.1 (0.3)	**0.017**
Chloride (mmol/L)	−1.5 (2.3)	0.6 (1.9)	<**0.001**	−2.1 (2.8)	−0.1 (1.9)	**0.006**
Calcium (mg/dL)	0.0 (0.3)	0.1 (0.2)	0.186	0.0 (0.4)	0.1 (0.2)	0.269
Phosphorus (mg/dL)	−0.1 (0.2)	0.3 (0.3)	<**0.001**	−0.3 (0.3)	0.0 (0.3)	<**0.001**
pH	0.02 (0.03)	−0.04 (0.04)	<**0.001**	0.02 (0.03)	−0.04 (0.04)	<**0.001**
Sodium bicarbonate	−0.3 (2.7)	−4.0 (2.6)	<**0.001**	−0.8 (2.4)	−4.4 (2.6)	<**0.001**
Base excess (mmol/L)	0.1 (2.1)	−4.0 (1.9)	<**0.001**	−0.3 (1.8)	−4.4 (2.1)	<**0.001**
Osmolality (mOsm/kg)	−2.1 (4.6)	−1.6 (4.4)	0.669	−3.3 (5.1)	−2.9 (3.6)	0.732
Magnesium (mg/dL)	−0.1 (0.1)	0.0 (0.1)	<**0.001**	−0.1 (0.1)	−0.1 (0.1)	0.120
White blood cells (/μL)	230 (725)	261 (784)	0.884	997 (1809)	1951 (1869)	0.073
Red blood cells ($\times 10^4/\mu$L)	−12.5 (62.4)	13.7 (17.0)	0.052	−1.7 (18.1)	8.2 (13.9)	**0.035**
Hemoglobin (g/dL)	−0.1 (0.5)	0.0 (2.1)	0.896	−0.1 (0.6)	1.2 (5.4)	0.234
Hematocrit (%)	−0.2 (1.6)	1.3 (1.7)	**0.003**	−0.4 (1.9)	0.8 (1.4)	**0.013**
Neutrophils (%)	1.3 (5.9)	3.0 (8.3)	0.404	2.1 (7.6)	6.2 (10.0)	0.107
Lymphocytes (%)	−0.7 (4.4)	−2.0 (7.3)	0.432	−0.7 (5.7)	−4.0 (8.4)	0.108
Eosinophils (%)	−0.6 (0.6)	−0.8 (1.1)	0.367	−1.0 (0..8)	−1.3 (1.6)	0.422

Values are mean (SD), *P* value by Welch's *t*-test, two-side.

that 2 L PEG+ASC was not inferior to 4 L PEG-ELS in bowel cleansing, with better acceptability of 2 L PEG+ASC than 4 L PEG-ELS in an inpatient setting, using a split-dose regimen the evening before colonoscopy and the following morning. Ponchon et al. [17] showed that 2 L PEG+ASC produced a similar high degree of cleansing and superior acceptability and tolerability compared with 4 L PEG-ELS in an outpatient setting, using a single-dose regimen the evening before colonoscopy. Indeed, there was heterogeneity among previous trials [9–20] regarding variations in the timing of bowel preparation, in the dosage schedule, in dietary instruction prior to and during the preparation, in diverse uses of the bowel preparation scale, and in the use of different adjuvants; these may all have contributed to those results. However, recently, Xie et al. [33] reported a meta-analysis of randomized controlled trials of low-volume PEG+ASC versus standard-volume PEG-ELS as bowel preparations for colonoscopy. In this report, eleven randomized controlled trials were identified for analysis; Xie et al. [33] demonstrated that the low-volume PEG+ASC achieved noninferior efficacy for bowel cleansing, was more acceptable to patients, and produced fewer side effects than the standard-volume PEG-ELS.

The present study showed no significant difference in bowel cleansing efficacy between the PEG+ASC group and the PEG-ELS group. However, the use of irrigation fluid seemed to be more frequent in the PEG+ASC group than in the PEG-ELS group. One of the reasons may have been foam and bubbles in the colonic lumen. In the present study, we evaluated the overall mucosal visibility that defined the amount of bubbles and foam. Although there were no significant differences between both groups, bubbles and foam may influence the results of the use of irrigation fluid. Several PEG-ELS formulations have added simethicone, which is an oral antifoaming agent that decreases bloating, abdominal discomfort, and abdominal pain by promoting the clearance of excessive gas along the gastrointestinal tract by reducing the surface tension of air bubbles [14, 22]. The combination of simethicone with PEG+ASC may be one of the choices for improving the efficacy, acceptability, and tolerability of PEG+ASC.

Patients favor preparations that are low in volume, are palatable, and have easy-to-complete regimens. However, contrary to our expectations, the present study found no significant difference in the comparison of taste between the two regimens, in contrast to the results of a previous study [9, 17]. One of the reasons is that most of this study's patients had already experienced bowel preparation of 2 L PEG-ELS in the previous 6 months, and they could get used to the flavor of PEG-ELS. Another reason is that patient compliance and acceptability may not be dependent on palatability, but rather the amount of PEG-ELS. In the present

Table 6: Differences in laboratory results between baseline and 3 hours and 7 hours after taking gut lavage solution in patients aged 70 years or more.

	Difference between 3 hrs and baseline			Difference between 7 hrs and baseline		
	PEG-ELS	PEG-ASC	*P*-value	PEG-ELS	PEG-ASC	*P*-value
Total protein (g/dL)	0.1 (0.4)	0.2 (0.5)	0.283	0.1 (0.3)	0.2 (0.4)	0.414
Albumin (g/dL)	0.1 (0.3)	0.1 (0.3)	0.420	0.1 (0.2)	0.1 (0.3)	0.817
Glucose (mg/dL)	−6.4 (8.4)	−4.4 (19.0)	0.627	−8.6 (16.6)	1.4 (23.1)	0.085
BUN (mg/dL)	−0.5 (1.1)	0.0 (1.2)	0.146	−0.7 (1.8)	0.3 (1.8)	0.055
Creatinine (mg/dL)	−0.01 (0.06)	0.03 (0.05)	**0.009**	0.00 (0.09)	0.00 (0.05)	0.828
ALT (U/L)	2.4 (2.0)	3.2 (5.3)	0.485	1.8 (1.9)	3.7 (6.1)	0.152
Sodium (mmol/L)	−0.6 (1.6)	−0.4 (1.4)	0.642	−0.7 (1.9)	−1.4 (1.9)	0.194
Potassium (mmol/L)	−0.1 (0.3)	0.0 (0.2)	0.070	−0.3 (0.3)	−0.2 (0.3)	0.130
Chloride (mmol/L)	−1.6 (1.3)	0.8 (1.4)	**<0.001**	−2.3 (2.2)	−0.3 (2.0)	**0.001**
Calcium (mg/dL)	−0.1 (0.3)	0.0 (0.4)	0.064	−0.1 (0.4)	0.0 (0.3)	0.417
Phosphorus (mg/dL)	0.0 (0.2)	0.3 (0.2)	**<0.001**	−0.2 (0.3)	0.0 (0.2)	**0.002**
pH	0.01 (0.03)	−0.03 (0.03)	**<0.001**	0.01 (0.03)	−0.02 (0.03)	**0.004**
Sodium bicarbonate	0.1 (2.5)	−3.9 (2.2)	**<0.001**	−0.4 (2.1)	−4.3 (2.1)	**<0.001**
Base excess (mmol/L)	0.4 (1.8)	−3.7 (2.1)	**<0.001**	−0.1 (1.7)	−3.9 (2.1)	**<0.001**
Osmolality (mOsm/kg)	−0.8 (6.1)	−1.1 (2.8)	0.815	−2.2 (3.9)	−2.7 (4.1)	0.643
Magnesium (mg/dL)	−0.1 (0.1)	0.0 (0.1)	**0.002**	−0.1 (0.1)	−0.1 (0.1)	0.787
White blood cells (/μL)	231 (1327)	−153 (789)	0.220	1223 (1024)	1489 (1021)	0.361
Red blood cells ($\times 10^4/\mu$L)	−0.9 (17.4)	5.2 (21.3)	0.277	−0.2 (19.6)	7.5 (19.6)	0.175
Hemoglobin (g/dL)	−0.1 (0.6)	0.0 (0.7)	0.458	−0.1 (0.6)	0.0 (0.6)	0.421
Hematocrit (%)	−0.1 (1.7)	0.9 (2.5)	0.103	−0.1 (2.0)	1.0 (2.7)	0.105
Neutrophils (%)	2.2 (5.8)	4.5 (7.0)	0.203	−0.8 (5.8)	2.6 (6.3)	0.052
Lymphocytes (%)	−1.2 (4.8)	−3.6 (6.3)	0.151	2.5 (4.7)	−0.4 (5.0)	**0.037**
Eosinophils (%)	−0.7 (0.7)	−0.6 (0.7)	0.621	−0.9 (1.0)	−1.0 (1.0)	0.669

Values are mean (SD), *P* value by Welch's *t*-test, two-side.

study, the reduction volume of PEG-ELS was only 0.5 L. However, there was much difference in the reduction volume as 2 L in previous studies [9–11, 13–20]. The reduction of the volume of PEG-ELS, regardless of the dose, despite being partly compensated by additional PEG-free clear liquid, must lead to improvement in patient acceptability of the bowel preparation. Better compliance, combined with the laxative effect of ascorbic acid, may account for the similar bowel preparation efficacies between the lower-volume PEG-ELS and the standard-volume PEG-ELS.

We demonstrated that the time to first defecation and the completion time for bowel preparation were significantly shorter in the 1.5 L PEG-ASC group than in the 2 L PEG-ELS group. Although the differences of shorted times are 15 minutes and 22 minutes, respectively, these saving times may lead to the improvement of patient acceptability in PEG-ASC group. Furthermore, these results will also provide a merit in saving time for the medical staffs. However, they should pay attention to patients because some patients in PEG-ASC group feel a need to evacuate their bowels rapidly compared to patients in PEG-ELS group.

PEG-ELS has been used worldwide since 1980 because of its well-established safety profile [4, 34, 35]. Because PEG-ELS is isotonic and electrolyte-balanced, there is little change in patient hydration and electrolytes in spite of the large volumes required [4, 34, 35]. On the other hand, although ascorbic acid is known to be safe even when taken in large doses [36, 37], there is concern about the occurrence of dehydration and electrolyte disturbance during bowel preparation with PEG+ASC compared with PEG-ELS, especially in elderly patients, because ascorbic acid possesses cathartic activity. Therefore, we evaluated standard biochemical and hematological parameters before starting the preparation (baseline) and at 3 hrs and 7 hrs after baseline. The usual time period to perform colonoscopy is seven hours or more, after starting the ingestion of the lavage solution. In our hospital, conventional colonoscopy for outpatients is conducted in the afternoon, with morning bowel cleansing. After finishing the outpatient colonoscopies, we prepare for endoscopic treatment of inpatients. During the waiting time after bowel preparation, we encourage patients to drink clear liquids to prevent dehydration. In our hospital, all endoscopic treatments for colorectal neoplasms are planned with admission the day before endoscopic treatment.

It was notable how many biochemical and hematological parameters had changed significantly after ingestion of the cleansing solutions, not only with PEG+ASC but also with PEG-ELS. Most of these changes continued until 7 hours after taking the bowel preparation. However, with the exception of sodium bicarbonate in the PEG+ASC group, all hematological and biochemical parameters varied within the normal range. The addition of electrolytes and ascorbic

acid to high molecular weight PEG-ELS may compensate for those changes, reducing the risk of electrolyte disturbances that can occur with other types of bowel preparation. However, there were distinctive biochemical changes in both groups. In the 2 L PEG-ELS group, electrolytes such as sodium and chloride, as well as glucose, had a tendency to decrease compared with the 1.5 L PEG+ASC group. On the other hand, in the 1.5 L PEG+ASC group, dehydration parameters such as total protein, albumin, and hematocrit had a tendency to increase, and acid-base balance parameters such as pH, sodium bicarbonate, base excess, and plasma osmolality had a tendency to decrease compared with the 2 L PEG-ELS group. Particularly, the sodium bicarbonate value in the 1.5 L PEG+ASC group decreased to beyond the lower normal range. These significant changes might be related to the composition of 2 L PEG-ELS and 1.5 L PEG+ASC. PEG+ASC contains ascorbic acid and sodium ascorbate instead of sodium sulfate that is present in PEG-ELS. These differences in composition produce hypertonia and higher acidity in PEG+ACS compared with PEG-ELS.

We also evaluated the differences in hematological and biochemical parameters between subjects younger and older than 70 years. There were no significant differences between the two age groups in any parameters before and after taking PEG-ELS and PEG+ASC. In both age groups, there was a tendency toward dehydration in the 1.5 L PEG+ASC group compared with the 2 L PEG-ELS group, showing that adequate hydration is needed. Although iso-osmotically balanced, PEG-ELS nevertheless has the ability in rare patients to induce hypovolemia combined with dysnatremia due to diarrhea, vomiting, and inadequate hydration during preparation. Hyponatremic hypovolemia can occur when PEG-ELS-induced volume loss results in an upregulation of arginine vasopressin, causing the patient to retain more free water than sodium [38]. When patients are unable to compensate for intestinal losses (such as the elderly with diminished thirst sensation) hypernatremic hypovolemia can occur [39]. Needless to say, the same warning is needed when using PEG+ASC.

The key strength of this study lies in its design. Randomization was stratified by age (<70 years or ≥70 years), and hematological and biochemical parameters were compared using blood samples in each age group, especially with respect to the safety profile of each regimen. We demonstrated significant statistical changes in the hematological and biochemical parameters after both types of preparation regardless of patient age, which were not clinically significant.

There are several limitations to consider in interpreting the results of this study. First, it was conducted in a single hospital with a small number of patients, that is, in inpatients scheduled to undergo endoscopic treatment. We obtained blood samples at baseline, and 3 hours and 7 hours after the patients took the preparation solution. Second, this study was designed to be conducted in an inpatient setting to maximize compliance. These features were incorporated to minimize bias and give high rates of good bowel preparation in both groups. The patients received their bowel preparation from a nurse who supervised its consumption, which probably provided a more accurate measure of compliance than when patients take the preparation at home. Therefore, the compliance rates with the bowel preparation solution in this study are probably higher than those normally achieved in routine outpatient use. Third, most of this study's patients had already experienced bowel preparation of 2 L PEG-ELS in the previous 6 months that may favor PEG+ASC as a new method and may lead to better acceptability in PEG-ASC. Fourth, we evaluated the efficacy, acceptability, tolerability, and safety of 1.5 L PEG+ASC compared with 2 L PEG-ELS; the results may not be applicable to patients in Western Hemisphere because 3-4 L of PEG-ELS has been used in there. Finally, we could not record any additional fluid intake during colonoscopy preparation, which may have influenced the grading of tolerance and acceptability. However, we do not believe that the additional fluid consumption contributed significantly to the efficacy in each group, as much of the excess fluid is absorbed in the upper gastrointestinal tract and excreted via the urinary system. Ell et al. [9] showed that the bowel cleaning efficacy is unrelated to additional fluid ingestion.

In conclusion, the present study demonstrated that patient acceptability was superior with the 1–5 L PEG+ASC regimen than with the 2 L PEG-ELS regimen; however, tolerability, safety, and bowel cleansing were similar in both groups. No significant differences in the safety profile were found between subjects aged less than 70 years and those aged 70 years or more; nevertheless, regardless of age, proper hydration is needed throughout the bowel preparation process.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 434039, 6 pages
http://dx.doi.org/10.1155/2015/434039

Clinical Study

The Prevalence of Gastric Intestinal Metaplasia and Distribution of *Helicobacter pylori* Infection, Atrophy, Dysplasia, and Cancer in Its Subtypes

Sehmus Olmez,[1] Mehmet Aslan,[2] Remzi Erten,[3] Suleyman Sayar,[4] and Irfan Bayram[3]

[1]*Medical Faculty, Department of Gastroenterology, Yuzuncu Yil University, 65080 Van, Turkey*
[2]*Medical Faculty, Department of Internal Medicine, Yuzuncu Yil University, 65080 Van, Turkey*
[3]*Medical Faculty, Department of Pathology, Yuzuncu Yil University, 65080 Van, Turkey*
[4]*Dr. Ersin Arslan Public Hospital, Department of Gastroenterology, Gaziantep, Turkey*

Correspondence should be addressed to Sehmus Olmez; drsehmusolmez@gmail.com

Received 10 January 2015; Revised 23 February 2015; Accepted 12 March 2015

Academic Editor: Jithinraj Edakkanambeth Varayil

Objectives. Gastric intestinal metaplasia (IM) is frequently encountered and is considered a precursor of gastric adenocarcinoma. In the Van region of Turkey, gastric adenocarcinoma incidence is high but the prevalence of gastric IM is not known. *Helicobacter pylori* (*H. pylori*) infection is a main factor leading to atrophy, IM, and cancer development in the stomach. The aim of the current study was to investigate the prevalence of IM and its subtypes and the prevalence of *H. pylori* infection, atrophy, dysplasia, and cancer in gastric IM subtypes. *Materials and Methods*. This retrospective study was conducted on 560 IM among the 4050 consecutive patients who were undergoing esophagogastroduodenoscopy (EGD) with biopsy between June 2010 and October 2014. Clinical records and endoscopic and histopathologic reports of patients with IM were analyzed. *Results*. The prevalence of gastric IM was 13.8%. The prevalence of incomplete IM was statistically significantly higher than complete IM. Type III IM was the most frequent subtype. *Conclusions*. Gastric IM is a common finding in patients undergoing EGD with biopsy in this region. High prevalence of incomplete type IM, especially type III, can be associated with the high prevalence of gastric cancer in our region.

1. Introduction

The incidence of gastric cancer has declined over the past decades but gastric cancer is still the fourth most common cancer and second leading cause of cancer related death worldwide [1, 2]. Gastric cancer has two types: intestinal and diffuse types. The pathogenesis of the intestinal type of gastric cancer is related to precursor lesions such as chronic atrophic gastritis, intestinal metaplasia (IM), and adenoma/dysplasia. However, the pathogenesis of the diffuse type of gastric cancer is not well defined [3].

Helicobacter pylori (*H. pylori*) infection is associated with severe gastritis, chronic atrophic gastritis, and IM, as well as gastric cancer [4–6]. Severe atrophy accompanying IM is related to a particularly high risk in *H. pylori* infected patients [7]. Some clinical studies reported that IM improves or did not progress after *H. pylori* eradication therapy [8–10]. As a result, *H. pylori* eradication is one of the most promising approaches in gastric cancer prevention [11].

Gastric IM is defined as replacement of gastric mucosa by epithelium of intestinal morphology and is generally considered to be a precursor of gastric cancer [12]. Risk factors of IM are *H. pylori* infection, high salt intake, smoking, alcohol consumption, and chronic bile reflux [13]. Although there are several classifications of IM types, currently they are generally subclassified as complete (type I) or incomplete (types II and III) [14, 15]. The incomplete type of IM, particularly type III, has a higher gastric cancer risk than the complete type [13, 15].

The incidence of gastric cancer varies in different parts of the world [1, 2]. Although the incidence of gastric cancer in Turkey is not high, its incidence in the Van region is very high and gastric cancer is an important health problem [16, 17]. Gastric IM is known as an important risk factor for gastric

cancer. As far as we know, no data is available regarding its prevalence in the Van region of Turkey.

The aim of the current study was to investigate the prevalence of IM and its subtypes and the prevalence of *H. pylori* infection, atrophy, dysplasia, and cancer in gastric IM. We also compared the relationship between *H. pylori* infection and IM subtypes.

2. Materials and Methods

2.1. Subjects. This retrospective study was conducted with 560 consecutive IM patients among the 4050 patients who were undergoing esophagogastroduodenoscopy (EGD), between June 2010 and October 2014, at the Department of Gastroenterology of the Yuzuncu Yıl University Medical Hospital.

Data including age, gender, indication for EGD, and endoscopic findings and pathology reports (histopathologic features, presence of associated chronic gastritis, *H. pylori* atrophy, dysplasia, and cancer) were retrieved from electronic medical records.

Indications for EGD are as follows: gastric cancer alarming symptoms or cancer screening, abdominal pain, anemia, dyspeptic symptoms, reflux, and follow-up of Barrett's esophagus and other premalignant lesions. If more than one EGD was performed with the same patient, only the first examination was included in the study.

The exclusion criteria were patients undergoing gastric surgery and upper gastrointestinal bleeding and patients with lacking data.

Esophagogastroduodenoscopy was performed after local 10% lidocaine sedation with a Fujinon video endoscope (EG 530 WR, Tokyo, Japan). Mean biopsies number was 3.7 ± 1.6 [1–12], and if lesions were suspected to be cancerous, extra biopsies were taken from the suspected area.

Biopsy specimens were evaluated for the presence of atrophic gastritis and IM. *H. pylori* was determined using histology testing. Pathologists of the Yuzuncu Yıl University Medical Hospital examined all the specimens.

All the endoscopically biopsied materials are fixed with Holland solution, after routine tissue follow-up processes embedded into paraffin blocks and stained with hematoxylin and eosin. Specimens are stained with periodic acid-Schiff/Alcian Blue (PAS/AB) at pH 2.5 stain combination and High Iron Diamine Alcian Blue (HID-AB) at pH 2.5 stain. Subtypes of IM are determined according to Filipe et al. classification [18].

The specimens were fixed in formalin and assessed for *H. pylori* (by Giemsa staining), the degree of neutrophil infiltration, IM (by staining with hematoxylin and eosin), atrophy, dysplasia, and other lesions.

IM was identified by replacement of glandular epithelium with goblet cells. IM was classified into complete (type I) or incomplete types (types II and III).

Type I includes mature absorptive cells and goblet cells, while the incomplete type secretes sialomucins. In type I IM, in PAS/AB stain at pH 2.5, the goblet cells are positively stained; the cylindrical cells between them show no reaction in both combinations and have markedly brush borders.

Type II has few or absent absorptive cells, presence of columnar "intermediate" cells in various stages of differentiation secreting neutral and acid sialomucins and goblet cells secreting sialomucins or, occasionally, sulfomucins, or both. In type II IM, in PAS/AB stain at pH 2.5, goblet cells are positively stained; cylindrical cells contain PAS positively stained granules; in HID/AB pH 2.5, cylindrical cells show no reaction.

Type III is formed by columnar intermediate cells secreting predominantly sulfomucins and goblet cells secreting sialomucins, sulfomucins, or both. If more than one subtype of IM was present, the specimen was classified based on the predominant IM phenotype. In type III IM, in PAS/AB stain at pH 2.5, goblet cells and cylindrical cells are positively stained with AB and in HID AB stain at pH 2.5 combination is positively stained.

The study protocol was approved by the ethics committee of our university.

2.2. Statistical Analysis. Descriptive statistics for continuous variables (characteristics) were presented as mean, standard deviation, and minimum and maximum values while they were count and percent for categorical variables. Chi-square test was used to determine linear association between categorical variables. In addition, Fisher's exact test was performed to compare two proportions in groups. Statistical significance level was considered as 5% and SPSS (version 13.0) statistical program was used for all statistical computations.

3. Results

Among the 4050 consecutive patients who had gastric biopsies performed, 560 were found to have gastric IM. Patient mean age was 57 ± 15 years (range: 17–98 years). There were 227 (40.5%) females and 333 (59.5%) males. The most frequent indications for EGD included dyspepsia (41.2%), abdominal pain (25.4%), and reflux symptoms (13.4%). The most frequent gastric endoscopic findings were gastritis (nonerosive, erosive, atrophic, etc.) (75.3%), ulcer (6.1%), and cancer (3.9%). Demographic features, indication for endoscopy, and endoscopic findings of the study population are shown in Table 1. The majority of patients were 41–70 years of age.

The prevalence of gastric IM was 13.8% at our institution. Eight of the 560 patients did not have an IM type (1.4%); 46 patients (8.2%) had complete type IM (Type 1). Of the 506 (90.4%) incomplete IM patients, 179 (32%) patients had type II, 214 (38%) patients had type III, and 113 (20.2%) patients had an unidentified subtype (Figure 1). The prevalence of incomplete IM was significantly higher than complete IM ($P < 0.05$).

Of the 46 complete IM patients, 6 had atrophy, 8 had dysplasia, and 1 had cancer. Of the 505 incomplete IM patients, 113 had atrophy, 72 had dysplasia, and 17 had cancer (Table 2).

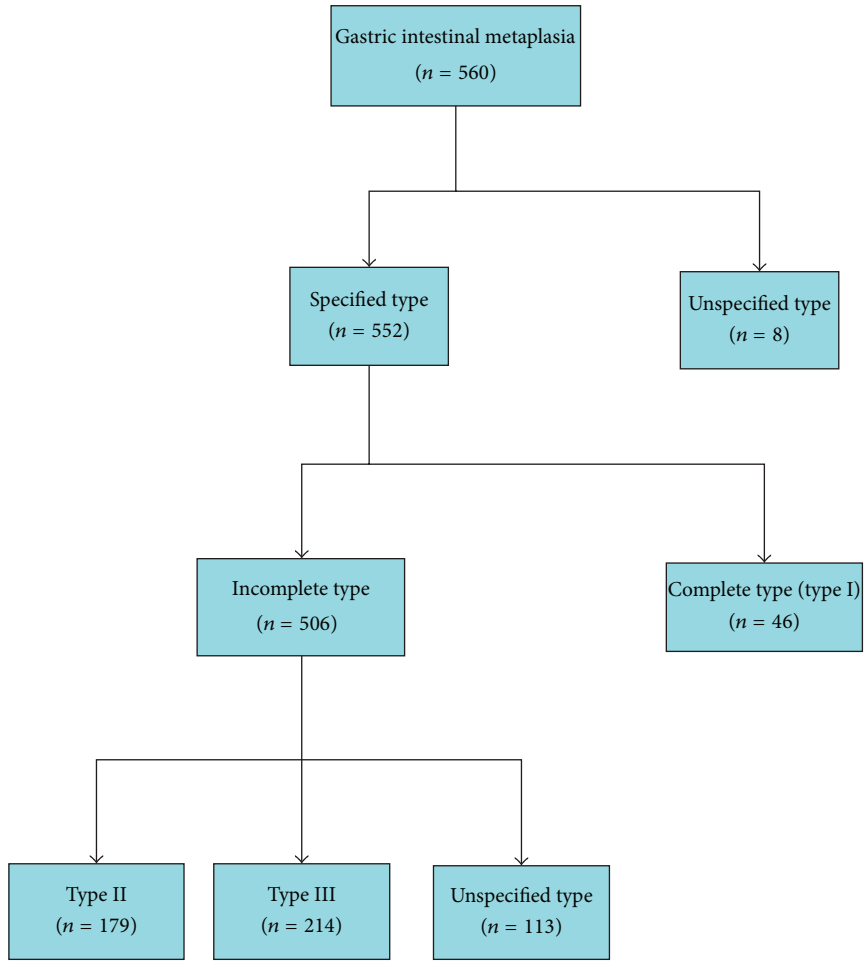

Figure 1: Flow diagram of intestinal metaplasia patients included in the study.

Prevalence of *H. pylori* infection was 38.6% in gastric IM. The relationship between *Helicobacter pylori* status and types of IM was assessed. No significant difference in prevalence of complete type and incomplete type IM was found with *H. pylori* status ($P > 0.05$) (Table 3).

4. Discussion

In the model of gastric carcinogenesis, *H. pylori* plays a pivotal role in causing chronic active gastritis. Chronic *H. pylori* induces gastritis and may progress over years through the sequential stages of atrophic gastritis, IM, and dysplasia to gastric adenocarcinoma [19, 20]. An important risk factor for gastric cancer development is the presence of premalignant changes of the gastric mucosa, such as IM, atrophy, and dysplasia [4, 11, 21].

The prevalence of gastric IM and atrophy in the general population is known to vary around the globe, mostly depending on *H. pylori* status [7, 22]. IM is a well-known risk factor for the development of gastric cancer [21, 22]. Diagnosis of atrophic gastritis, IM, and dysplasia is often ignored in routine clinical practice [23]. There are no widely accepted guidelines for the management of gastric IM [24]. The American Society for Gastrointestinal Endoscopy (ASGE) and The European Society of Gastrointestinal Endoscopy and other European academic societies have developed evidence-based guidelines for the management of patients with gastric IM [11, 25]. ASGE guidelines for endoscopic surveillance for gastric IM cannot be uniformly recommended but surveillance may be beneficial for patients at increased risk of gastric cancer due to ethnic background or family history [25]. Recently, according to the published guidelines for endoscopic management of precancerous conditions and lesions in the stomach, endoscopic surveys should be offered to patients with extensive atrophy and/or IM without subtyping every three years [11]. Several studies demonstrated that endoscopic histological follow-up in patients with IM is able to detect gastric cancer at an early stage with a considerable mortality reduction and scheduled endoscopic control could be cost-effective in IM patients [26–28]. According to some authors, complete IM is associated with a lower risk of gastric cancer; therefore, in the absence of other risk factors for gastric cancer, patients with complete IM do not need long-term endoscopic surveillance [13]. Due to the high prevalence of gastric cancer and *H. pylori* infection in our region, we

Table 1: Demographic features, indication for EGD, and EGD findings of patients with intestinal metaplasia.

	(n = 560)
Sex	
Male	227 (40.5%)
Female	333 (59.5%)
Age (years)	57.0 ± 15.3 (17–98)
Age groups	
<31	29 (5.2%)
31–40	56 (10%)
41–50	115 (18.8%)
51–60	144 (25.7%)
61–70	113 (20.2%)
71–80	87 (15.5%)
>80	26 (4.6%)
Indication for EGD	
Dyspepsia	231 (41.2%).
Abdominal pain	145 (25.9%)
GERD	75 (13.4%)
Cancer screening	29 (5.2%)
Others	80 (14.3%)
EGD findings	
Gastritis (nonerosive, erosive, atrophic, etc)	378 (67.5%)
Gastric ulcer	34 (6.1%)
Gastric cancer	22 (3.9%)
Others	126 (22.5%)

GERD: gastroesophageal reflux disease; EGD: esophagogastroduodenoscopy.

Table 2: Prevalence of atrophy, dysplasia, and cancer in types of intestinal metaplasia.

	Complete IM (N = 46) n (%)	Incomplete IM (N = 506) n (%)	Fisher's exact test P value
Atrophy	6 (13%)	113 (22.3%)	0.080
Dysplasia	8 (17.4%)	72 (14.2%)	0.586
Cancer	1 (2.2 %)	17 (3.4%)	0.999

IM: intestinal metaplasia; $P < 0.05$ statistically significant.

Table 3: Relationship between *Helicobacter pylori* status and types of intestinal metaplasia.

Helicobacter Pylori	Complete IM (N = 46) n (%)	Incomplete IM (N = 506) n (%)	Fisher's exact test P value
Negative	25 (54.3%)	314 (62.1%)	0.314
Positive	21 (45.7%)	192 (37.9%)	

IM: intestinal metaplasia; $P < 0.05$ statistically significant.

strongly recommend endoscopic surveillance of gastric IM without paying attention to subtype.

The prevalence of gastric IM was 13.8% in our study. The prevalence of gastric IM in the general population remains difficult to ascertain due to the asymptomatic nature of the lesion. There is a wide variation in the prevalence of gastric IM depending on the different methods used in studies and particularly the prevalence of *H. pylori* infection in the region. [22, 28]. Sonnenberg et al. conducted a large retrospective study of 78,985 patients undergoing EGD with biopsy across the United States and found that the prevalence of gastric IM was 7% [29]. Almouradi et al. reported that, among the 437 patients who had gastric biopsies performed, 66 were found to have gastric IM and they observed that the overall prevalence was 15% [30]. In Netherlands, the prevalence of IM in a large histopathology database was reported as 8% [21]. We think that the prevalence of IM may be higher in our region. This low prevalence of IM may derive from the insufficient number and inappropriate localization of biopsies.

In our study, the prevalence of incomplete type of IM (90.4%) was much higher than the complete type (8.2%). The type III IM was the highest subtype. Eriksson et al. reported that among 505 patients the total prevalence of IM was 19% and the prevalence of type III IM was 2.8%, type II IM was 4.4%, and type I IM was 11% [31]. Ozdil et al. reported that of 3301 consecutive dyspeptic patients 17.8% had IM. They observed that 86% had complete and 14% had incomplete IM [32].

The presence of incomplete type IM, especially type III, significantly increases the risk of gastric cancer as compared to type I and type II [28, 33–35]. High detection of incomplete type IM, particularly type III, may shed light on the high prevalence of gastric cancer in our region. The prevalence of incomplete type IM was considerably higher than in previous studies [36, 37].

Evidence on establishing the subtype of IM is limited and conflicting [24]. There are several problems when subtyping IM. There is the need for additional histochemical techniques and these techniques are far from being standardized. The current technique uses reagents such as High Iron Diamine Alcian Blue staining, which are toxic and potentially carcinogenic. Pathologically, subtyping is sometimes difficult because IM have a multifocal distribution within the stomach and IM areas may be small. As a result, sampling errors may be unavoidable. Also, different subtypes of IM frequently coexist. When there are different subtypes, it is unclear which should be considered for categorizing [12, 34]. Perhaps due to these reasons, in our study, a distinction of complete and incomplete types in eight of 560 patients and a distinction of type II and type III in 113 patients could not be taken into account.

In our study, we observed that gastric IM was higher in older than in younger patients. Several studies revealed that age ≥50 years was an independent risk factor for IM, which is consistent with previous studies reporting that the incidence of IM increases with age [25, 29, 36].

In a study regarding *H. pylori* infection in Turkey, Özden et al. found *H. pylori* positivity to be 81% in the general population [38]. In our study, the prevalence of *H. pylori* infection was 38.6% in gastric IM and no significant difference in prevalence of complete type and incomplete type IM was found with *H. pylori* presence. With and without atrophy, IM may cause lower diagnostic accuracy of *H. pylori* with

histologic examination [13]. In addition, decreased sensitivity of the rapid urease test is observed in subjects taking proton pump inhibitors, so other diagnostic tests, including serology or stool antigen, should be considered [13, 37]. *H. pylori* eradication is one of the most promising approaches in gastric cancer prevention [11]. However, whether gastric atrophy and IM are reversible after the eradication of *H. pylori* remains controversial [22, 39], since in some studies eradication of *H. pylori* has not been effective in IM regressions [8–10, 26]. However, several studies have shown that eradication of *H. pylori* infection significantly retarded progression of gastric IM and therefore *H. pylori* eradication has been proposed [40–43].

This retrospective study design has several potential limitations. Firstly, biopsies may not have been taken in adequate number and from the same part of the stomach. Secondly, results of the study may be affected by the lack of medical history such as information about proton pump inhibitors or antibiotic usage in some patients. Finally, diagnosis of *H. pylori* infection was only made by histological examination.

In conclusion, this study indicated that gastric IM is a common finding in patients undergoing EGD with biopsy. In addition, we found that the prevalence of incomplete IM was statistically significantly higher than complete IM. In our study population, type III IM was the most frequent subtype. High prevalence of the incomplete type and notably type III may be associated with a high prevalence of gastric cancer in our region. *H. pylori* was detected in 38.6% of gastric IM patients in Turkey. To further establish the relationship between IM and gastric cancer in our region, large prospective, randomized, and multicenter studies are needed.

Conflict of Interests

The authors declared no conflict of interests.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 343642, 12 pages
http://dx.doi.org/10.1155/2015/343642

Research Article

Anti-Inflammation Property of *Syzygium cumini* (L.) Skeels on Indomethacin-Induced Acute Gastric Ulceration

Lanchakon Chanudom and Jitbanjong Tangpong

Biomedical Sciences, School of Allied Health Sciences and Public Health, Walailak University, Nakhon Si Thammarat 80160, Thailand

Correspondence should be addressed to Jitbanjong Tangpong; njibjoy@yahoo.com

Received 9 December 2014; Revised 23 February 2015; Accepted 11 March 2015

Academic Editor: Ilja Tacheci

Indomethacin, nonsteroidal anti-inflammatory drug (NSAIDs), induced gastric damage and perforation through the excess generation of reactive oxygen species (ROS). *Syzygium cumini* (L.) Skeels is commonly used as a medicinal plant and is claimed to have antioxidant activities. The effects of *Syzygium cumini* (L.) Skeels aqueous extract (SCC) on antifree radical, anti-inflammation, and antiulcer of SCC on indomethacin induced acute gastric ulceration were determined in our study. Scavenging activity at 50% of SCC is higher than ascorbic acid in *in vitro* study. Mice treated with indomethacin revealed mucosal hemorrhagic lesion and inhibited mucus content. Pretreatment with SCC caused discernible decrease in indomethacin induced gastric lesion and lipid peroxide content. In addition, oxidized glutathione (GSSG), glutathione peroxidase (GPx), nitric oxide (NO) levels, and gastric wall mucus were restored on acute treated mice model. Indomethacin induced inflammation by activated inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) proinflammatory cytokines to release large amount of ROS/RNS which were ameliorated in mice pretreatment with SCC. SCC showed restoration of the imbalance of oxidative damage leading to amelioration of cyclooxygenase enzyme (COX). In conclusion, SCC acts as an antioxidant, anti-inflammation, and antiulcer against indomethacin.

1. Introduction

Syzygium cumini (L.) Skeels from Myrtaceae family is a native tree of the tropics, originally from India and Southeast Asia. It is widespread in north, northeast, and south of Thailand and used as a popular treatment against various diseases. The bark, fruits, seeds, and leaves of *Syzygium cumini* (L.) Skeels were used for the treatment of diabetes and administered in various pharmaceutical preparations in Brazil [1]. Seeds have shown hypoglycemic and antioxidant activities. The bark is also used for dysentery and diarrhea. Moreover, *Syzygium cumini* (L.) Skeels has been shown to have sedative and anticonvulsant effects and a potent central nervous system depressant effect [2] and this plant is rich in compounds containing anthocyanins, glucoside, ellagic acid, isoquercetin, kaempferol, and myricetin. The leaves are claimed to contain acylated flavonol glycosides, quercetin, myricetin, myricetin 3-0-4-acetyl-L-rhamnopyranoside, triterpenoids, esterase, galloyl carboxylase, and tannin [3].

Among the commonly used nonsteroidal anti-inflammatory drugs (NSAIDs), indomethacin possesses the highest ulcerogenic potential to humans. Nevertheless, it is still extensively used in obstetrics to delay uterine contractions and in the neonatal unit to facilitate patent ductus arteriosus closure. Also, it is most preferred for various mechanistic studies on NSAID physiology. Inhibition of the cyclooxygenases (COXs) and the associated reduced prostaglandin (PG) synthesis were believed to be the major reasons for gastric pathogenesis caused by NSAIDs including indomethacin. However, accumulated evidence suggested that multiple other COX-independent factors play equally important roles in the process. Indomethacin is a known inducer of reactive oxygen and nitrogen species in animal models [4], which may contribute to mucosal injury. It is becoming increasingly apparent that leukocyte-endothelial cell interaction, caused by various adhesion molecules [5], is a critical and early event in the pathogenesis of NSAID-induced gastropathy.

The treatment of ulceration on NSAIDs induced gastric damage used the existing synthetic antiulcer drugs which confer mild-to-severe side effects and are expensive especially for the rural people and some of the antiulcer drugs are known to exert their action via antioxidative activity [6]. Therefore, the traditional plants which have high antioxidants actions might provide inexpensive and nontoxic medications for people. To find out this problem, our research was designed and aimed to determine the total phenolic and flavonoid contents, scavenging capacities in cell free system, anti-inflammation, and antiulcer action against indomethacin NSAIDs induced gastric damage of *Syzygium cumini* (L.) Skeels aqueous crude extracts (SCC). Thus, our experiment may highlight the potential of SCC as a new source of natural antioxidants and anti-inflammation and as a peptic ulceration inhibitor.

2. Materials and Methods

2.1. Plants. *Syzygium cumini* (L.) Skeels was collected from different natural habitats areas at Nakhon Si Thammarat province, Thailand. The fresh leaves were rinsed with distilled water. To prepare aqueous leaf extracts of *Syzygium cumini* (L.) Skeels or SCC, *Syzygium cumini* (L.) Skeels was chopped and homogenized into distilled water in a blender for 1 min. After that the suspension was filtered by Whatman number 1 and then lyophilized by freeze dryer at −20°C for 20 h. The powder was kept at −30°C until used.

2.2. Determination of Total Phenolic and Flavonoid Contents of SCC. The total phenolic of SCC was determined with the Folin-Ciocalteu reagent using the method of Lister and Wilson [7] as slightly modified on this study. Briefly, 20 μL of sample in triplicate and 100 μL of 2 N Folin-Ciocalteu's reagent were added and incubated at room temperature for 5 min. 300 μL of Na_2CO_3 (25% w/v) was mixed and incubated at 45°C for 30 min. The absorbance of SCC was read at 765 nm using UV-visible spectrophotometer. Results were expressed as microgram of gallic acid equivalent per milliliter (mgGAE/mL).

Aluminum chloride colorimetric method was used for flavonoid determination [8]. 1 mL of SCC (100 μg/mL) was mixed with 3 mL of methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1 M $C_2H_3KO_2$, and 5.6 mL of distilled water and then incubated at room temperature for 30 min and the absorbance of the reaction mixture was measured at 415 nm with UV-visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at various concentrations in methanol. The flavonoid content was calculated and expressed in microgram quercetin per milliliter ($\mu g_{quercetin}$/mL).

2.3. Determination of Scavenging Activities of SCC. The free radical scavenging of SCC at various concentrations (6.25–2,000 μg/mL) was determined by ABTS cation decolorization assay [9] and results were expressed as a percentage of the scavenging. Hydroxyl radical assay according to the Fenton-type reaction was measured. The reaction mixture contained 1 mL of 0.1 mM methyl violet, 0.5 mL of 5 mM $FeSO_4$, 0.5 mL of 1% H_2O_2, and 2 mL of Tris buffer (pH 4.0). The reaction volume is 10 mL. 0.5 mL of SCC at a different concentration was added. The absorbance of the reaction mixture was measured at 565 nm by a spectrophotometer. The absorbance with SCC was measured as A_s, the absorbance without SCC was A_0, and absorbance without $FeSO_4$ and H_2O_2 was determined as A. Scavenging activity of SCC on hydroxyl radical was calculated according to the following formula: $[(A_s - A_o)/(A - A_o)] \times 100$.

The scavenging activity of the SCC on superoxide anion was calculated by comparing ΔA_1/min (without SCC) and ΔA_2/min (with SCC) of the pyrogallol system; 100 μL of 3 mM pyrogallol and 3 mL of Tris buffer (pH 8.2) were mixed with 0.5 mL of SCC at different concentrations and the autooxidation rate of pyrogallol was measured by determining the changes in the absorbance at 325 nm in 4 min by UV-visible spectrophotometer. The absorbance with SCC was A_2 and the absorbance without SCC was determined as A_1 following the formula $[(\Delta A_1 - \Delta A_2)/\Delta A_1] \times 100$ [10]. Ascorbic acid was used as natural antioxidant compound to compare the free radical $ABTS^{\bullet+}$, hydroxyl, and superoxide radical scavenging action with SCC. Nitric oxide scavenging capacity assay was measured at 550 nm using the Griess reagent [11]. Gallic acid was used as a reference of natural antioxidant compound to compare the scavenging action on nitric oxide radical. The scavenging capacities at 50% (SC_{50}) of free radical $ABTS^{o+}$, hydroxyl, superoxide, and nitric oxide radicals were calculated.

2.4. The Protective Effects of SCC on Acute Gastric Ulcer Induced by Indomethacin In Vivo

2.4.1. Animals. The mice were bred at Mahidol University, Bangkok, Thailand. These were procured after obtaining clearance from the respective Animal Committee of the Walailak University, Thailand, and were handled following International Animal Ethics Committee Guidelines. Male mice (25–30 g body weight), five weeks old, were divided into seven groups of six mice each and maintained and kept in controlled environment, at a constant temperature (23 ± 2°C) and humidity (60 ± 10%) and a 12 h light/dark cycle. Mice were acclimatized for one week before any experimental procedures and were allowed standard rat chow and tap water *ad libitum*.

2.4.2. Acute Induction of Indomethacin Gastric Mucosal Lesions and Treatments. According to Santucci et al. [12], mice were conducted with seven independent groups ($n = 6$ per group) and orally treated as follows:

group 1: untreated control (phosphate buffer solution: PBS),

group 2: PBS + indomethacin (20 mg/kgBW),

group 3: SCC (100 mg/kgBW) + indomethacin (20 mg/kgBW),

group 4: SCC (250 mg/kgBW) + indomethacin (20 mg/kgBW),

group 5: SCC (500 mg/kgBW) + indomethacin (20 mg/kgBW),

group 6: omeprazole (100 mg/kgBW) + indomethacin (20 mg/kgBW),

group 7: vitamin C (100 mg/kgBW) + indomethacin (20 mg/kgBW).

20 mg/kgBW of indomethacin suspended in an aqueous solution of sodium bicarbonate (5%) was administered after one hour of pretreatment of group 2 to group 7. Four hours later, the stomachs of the animals were removed. The ulcerated portions of the stomach were sectioned after fixing in 10% formal saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 μm onto a glass slide; the pathological changes of gastric tissue was evaluated with haematoxylin-eosin staining and examined under a light microscope. The damage scores, inhibition percent, mucin contents, and oxidative and inflammation parameter of all groups were measured.

The gastric ulcer was evaluated by measuring the length of ulcer in mm scale. Ulcer index (UI) was calculated from the total of gastric ulcer area (mm) divided with total mice in each group. Inhibition percent (% inhibition) was calculated following the formula

$$\%\text{Inhibition} = \left(\frac{(\text{UI of control group} - \text{UI of treatment group})}{\text{UI of control group.}}\right) \times 100. \quad (1)$$

2.4.3. Determination of Gastric Mucus Content. The mucus bound to the epithelial surface (μg/g tissue) was performed according to the method of Corne et al. [13]. The glandular portion of the stomach was removed and immersed in 0.1% (w/v) alcian blue in sucrose (160 mmol/L) buffered with sodium acetate (50 mmol/L, pH 5.8). The unbound dye was eluted twice with sucrose (250 mmol/L) and the mucus bound dye was extracted with $MgCl_2$ (500 mmol/L, pH 6). The solution was then shaken with an equal volume of diethyl ether, centrifuged at 3000 g for 10 min, and the amount of alcian blue in the aqueous phase was measured spectrophotometrically at 580 nm. Pathological study showed higher accurate data more than spectrophotometry.

2.4.4. Determination of Oxidative Damage

(1) Determination of Lipid Peroxides. The thiobarbituric acid reaction of Uchiyama and Mihara [14] was adopted for estimation of lipid peroxides level, using malondialdehyde as a standard. Gastric mucosal homogenates 1% (v/v) orthophosphoric acid and 0.6% (w/v) thiobarbituric acid were added; mixtures were then boiled for 45 min at 100°C. After cooling, the colored product was extracted by n-butanol, vortexed, and centrifuged at 3000 g for 15 min. The absorbance of the upper layer was read spectrophotometrically at 535 and 520 nm. The difference in absorbance was calculated as gastric mucosal lipid peroxides level and expressed as nmol/mg tissue malondialdehyde.

(2) Determination of Oxidized Glutathione (GSSG). Oxidized glutathione was determined following Rolland [15]. The gastric tissue was homoginated and diluted to 10 folds; 10 μL of EDTA and 10 μL of NADPH were added and mixed and the baseline level of NADPH absorbance was recorded. For measurement of standard GSSG solution, 1 mL sample was replaced by 1 mL buffer and standard GSSG at volume containing 5 nmol. The oxidized glutathione levels were present as ng/mg protein.

(3) Determination of Glutathione Peroxidase (GPx). The reaction mixture consisted of 100 μL of buffer, 20 μL of GSH, 100 μL of GR, 100 μL of NADPH, 10 μL of homoginated gastric tissue, 10 μL of NaN_3, and 660 μL of distilled water. This mixture was warmed at 37°C for 10 min and then 10 μL of t-butyl hydroperoxide was added. The OD at 340 nm was then followed. A blank value was obtained without any addition of substrates and was subtracted from the assay values. The activity of glutathione peroxidase was calculated from the change in optical density in 1 min and the molar extinction coefficient for NADPH at 340 nm of 6.22 nM/cm. The activity was expressed as U/g protein. Protein (mg/g tissue) was determined by Lowry et al. [16] method.

(4) Determination of Nitric Oxide Level. Nitric oxide (nmol/mg tissue) was quantified indirectly as nitrite/nitrate concentration using Griess reaction-dependent method [17]. Gastric mucosal homogenates were deproteinated with absolute ethanol for 48 h at 4°C and then centrifuged at 12000 g for 15 min at 4°C. To an aliquot of the supernatant vanadium trichloride 0.8% (w/v) in 1 M HCl was added for the reduction of nitrate to nitrite, followed by the rapid addition Griess reagent consisting of 0.1% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride and 2% (w/v) sulfanilamide in 5% (v/v) HCl and incubated for 30 min at 37°C. Mixtures were cooled and the absorbance at 540 nm was measured.

2.4.5. Determination of Inflammatory Parameter

(1) Tumor Necrosis Factor-Alpha (TNF-α) Assay. The plasma samples were collected from the mice. The levels of TNF-α in plasma were determined by ELISA assay following the manufacturer's instructions (Millipore, USA). Each sample was added in 96-well plate and incubated for 1 h with TNF-α detection antibody solution. Wells were washed 4 times with PBS assay buffer and conjugated with avidin-HRP A solution for 30 min, discarded, and washed to minimize background. 100 μL of substrate solution F was added and incubated for 15 min in the dark to form a complex with a stabilized chromogen. Stop the reaction by adding 100 μL of stop solution to get yellow color. Read absorbance at 450 nm within 30 min and subtract with the absorbance at 570 nm. The TNF-α concentration in the sample was calculated from the recombinant mouse TNF-α standard curve.

(2) Western Blot Technique for COX-1, COX-2, and iNOS Expression. The glandular part of the gastric mucosa, after being washed with PBS containing protease inhibitors, was minced and homogenized in a PBS buffer obtaining protease

inhibitor. After centrifugation at 14,000 g for 20 min at 4°C, the supernatant was collected, aliquoted, and kept at −80°C before use in the western blots. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20) containing 5% bovine serum albumin or skim milk and incubated overnight at 4°C with the appropriate primary antibody. The membrane was washed with TBST twice for 7 min and Tris-buffered saline (TBS). Then membrane was incubated with the appropriate peroxidase conjugated secondary antibody. The bands were detected using an enhanced chemiluminescence detection kit and quantified with respect to the bands of a suitable loading control, using the Kodak Gelquant software [18].

2.5. Statistical Analysis. Statistical comparisons were made using one-way ANOVA followed by Newman-Keuls multiple comparisons test. All results are given as mean ± S.E.M. of at least three separate experiments. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Total Phenolic and Flavonoid Contents of SCC. Total phenolic content of SCC was found to be increased from 134.44 ± 2.22–694.44 ± 3.64 μgGAE/mL at the concentration of 62.5–2000 μg/mL of SCC, respectively, depending on dose dependent manner. Flavonoid content was calculated from the regression equation of the quercetin calibration curve ($y = 0.023X + 0.118$). The total flavonoid content was increased from 0.21 ± 0.04–8.27 ± 0.10 $\mu g_{quercetin}$/mL at the concentration of 125–2000 μg/mL of SCC, respectively. The result of total phenolic and flavonoid contents are summarized in Figure 1(a) and the correlation between total phenolic and flavonoid contents was almost related and depended on the concentration of SCC at 0.93 of r^2 value (Figure 1(b)).

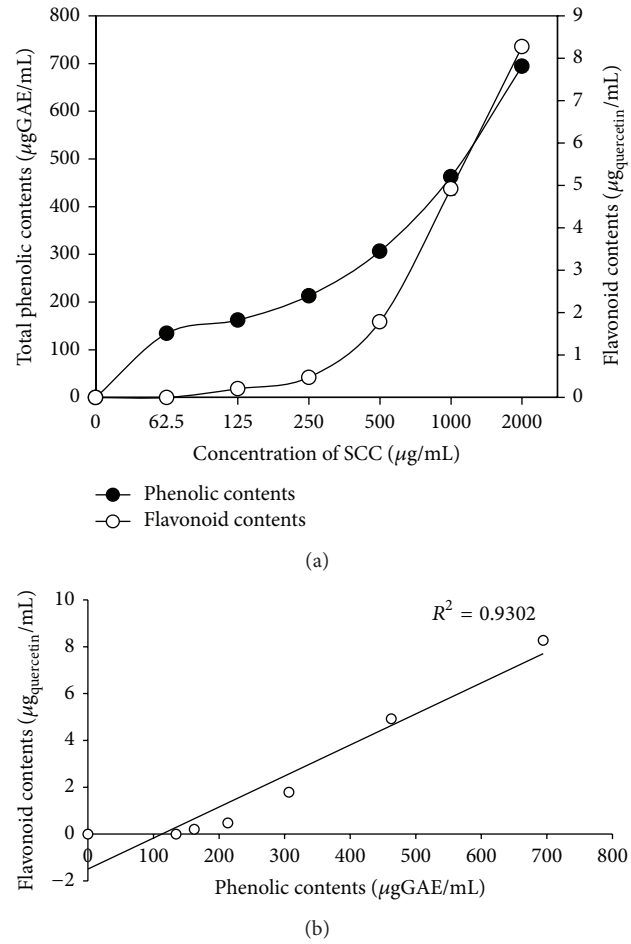

Figure 1: Total phenolic and flavonoid contents of SCC at various concentrations (a) and the correlation of phenolic and flavonoid contents of SCC (b).

3.2. Scavenging Activities of SCC. Scavenging activities at 50% (SC_{50}) values of SCC on free radical $ABTS^{\bullet+}$, hydroxyl, and superoxide radical were 87.79 ± 0.85, 766.62 ± 28.61, and 1,299 ± 25.13 μg/mL, respectively. This data indicated that increasing of SCC concentration was related to the increased radical scavenging activities, especially for scavenging on $ABTS^{o+}$ radical of SCC was comparable active than ascorbic acid (Figure 2(a)). The scavenging activities of SCC and ascorbic acid at the concentration of 62.5–2,000 μg/mL on hydroxyl radical were increased from 6.81 ± 1.00–52.09 ± 0.75 and 18.29 ± 2.14–69.04 ± 0.97%, respectively (Figure 2(b)) and superoxide radical scavenging activities of SCC and ascorbic acid at the concentration of 125–2,000 μg/mL were concentration dependent increasing from 2.21 ± 0.07–66.36 ± 1.82 and 19.42 ± 1.51–84.50 ± 4.58%, respectively (Figure 2(c)). Nitric oxide scavenging activities of SCC were measured and compared to gallic acid (Figure 2(d)). The scavenging activities of SCC and gallic acid at the concentration of 62.5–2,000 μg/mL on nitric oxide radical were increased from 4.15 ± 0.92–13.05 ± 0.96 and 7.39 ± 0.97–32.89 ± 0.81%, respectively. The SC_{50} of nitric oxide of SCC and gallic acid were not calculated because the scavenge action did not reach 50%; even the concentration of SCC and gallic acid was increased up to 8,000 μg/mL (data not shown).

3.3. Protective Effect of SCC on Indomethacin-Induced Gastric Ulceration

3.3.1. Ulcer Index (UI) and Inhibition Percent of SCC Treated Mice. The evaluation of gastric ulcer was measured by the length area in mm and calculated into UI values. The UI values of all groups were demonstrated in Figure 3(a). The indomethacin treated group was the highest and showed significant increase in UI values when compared with control ($P < 0.05$) at 445 ± 72.09.

SCC treated group at the concentration of 100–500 mg/kgBW significantly reduced the UI values when compared with indomethacin treated group and UI values of SCC at 100–500 mg/kgBW were 284.09 ± 50.82, 150 ± 22.82, and 85 ± 11.90, respectively. The SCC at 500 mg/kgBW was more potent than other compounds; it has shown about 80% decrease in UI when compared to indomethacin treated group.

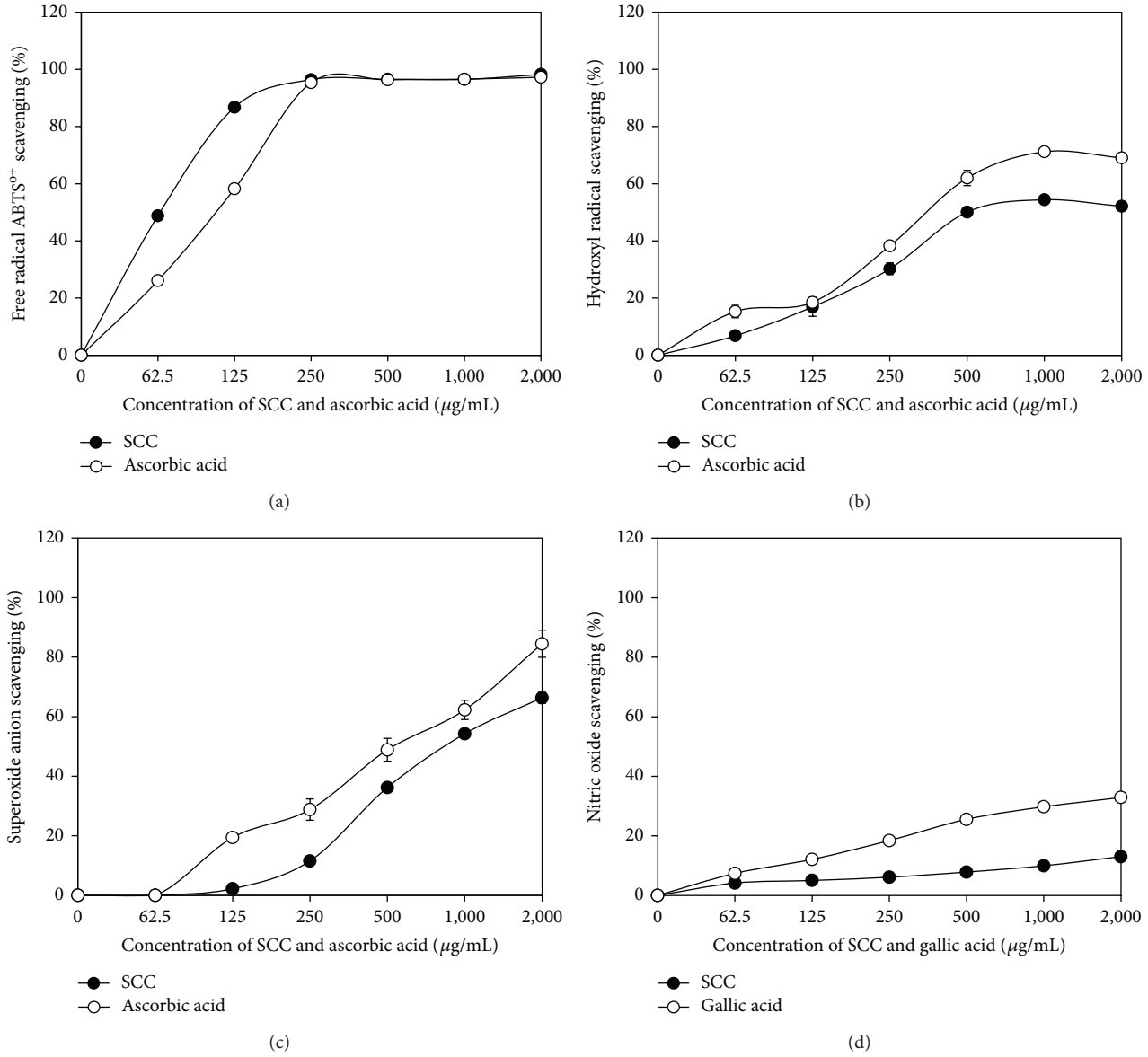

Figure 2: Scavenging activities of SCC, ascorbic acid, and gallic acid on free radical ABTS$^{\bullet+}$ radical cation (a), hydroxyl radical (b), superoxide radical (c), and nitric oxide radical (d).

Omeprazole and vitamin C at 100 mg/kgBW significantly decreased the UI values when compared with indomethacin treated group and had shown the UI values at 146.88 ± 31.49 and 127.27 ± 19.70, respectively.

UI values were calculated into inhibition percent by being compared with indomethacin treated group. SCC treated group at the concentration of 100–500 mg/kgBW inhibited the gastric ulcer at 36.16 ± 11.42, 66.29 ± 5.13, and 80.89 ± 2.67%, respectively (Figure 3(b)). Omeprazole and vitamin C at 100 mg/kgBW inhibited the ulceration by induction of the inhibition percent to 66.99 ± 7.08 and 71.39 ± 4.45%, respectively. The SCC, omeprazole, and vitamin C treated groups significantly increased inhibition percent when being compared with indomethacin treated group ($P < 0.05$). SCC at 500 mg/kgBW was the highest effective compound to protect the ulceration induced by 20 mg/kgBW indomethacin and significantly reduced the ulcer better than omeprazole and vitamin C treated group. The physiology of gastric tissue of each group was demonstrated in Figure 4(a). The dark areas in the photograph represented the area of ulceration. The indomethacin treated group had shown the severe ulcer area with many dark spot areas. However, when mice were treated with SCC, omeprazole, and vitamin C, the areas of ulcer were reduced in all groups. Specifically, when treated with SCC 500 mg/kgBW, the physiology of gastric tissue is not different from normal mice.

3.3.2. Pathology of Gastric Tissue of SCC Prevents Gastric Ulceration Induced by Indomethacin. Figure 4(b) demonstrated the pathology of gastric tissue after being treated

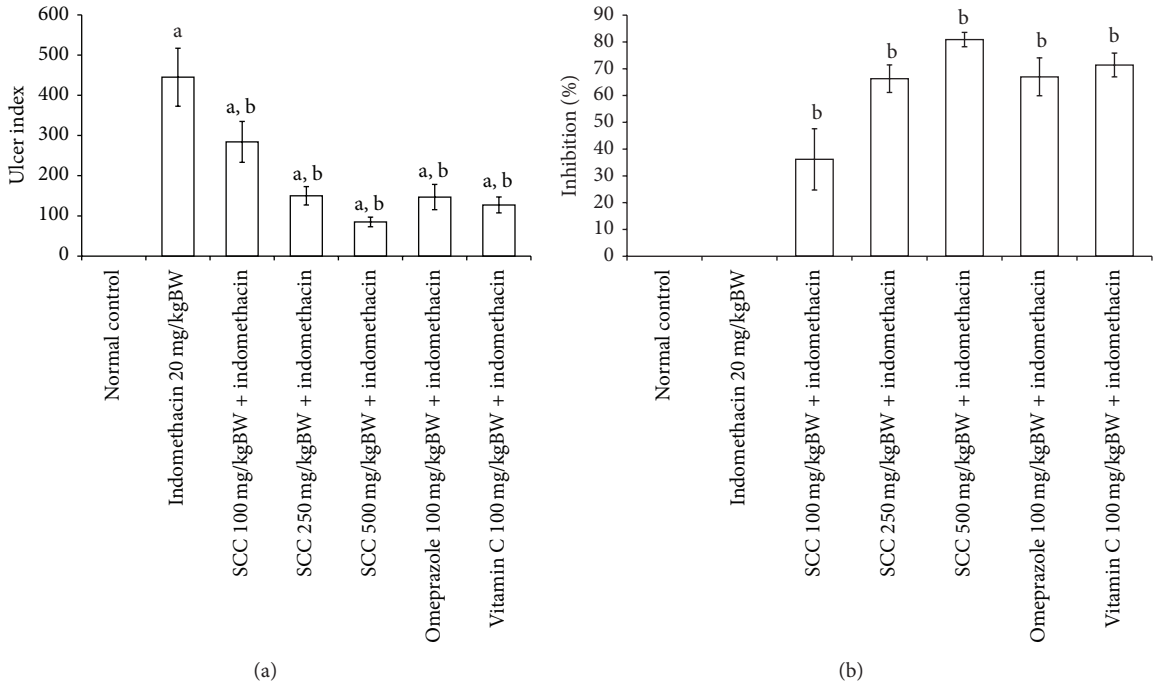

FIGURE 3: Ulcer index (a) and ulcer inhibition percent (b) of acute study. Data shown as mean ± S.E.M. a: $P < 0.05$ when compared with normal group and b: $P < 0.05$ when compared with indomethacin 20 mg/kgBW group.

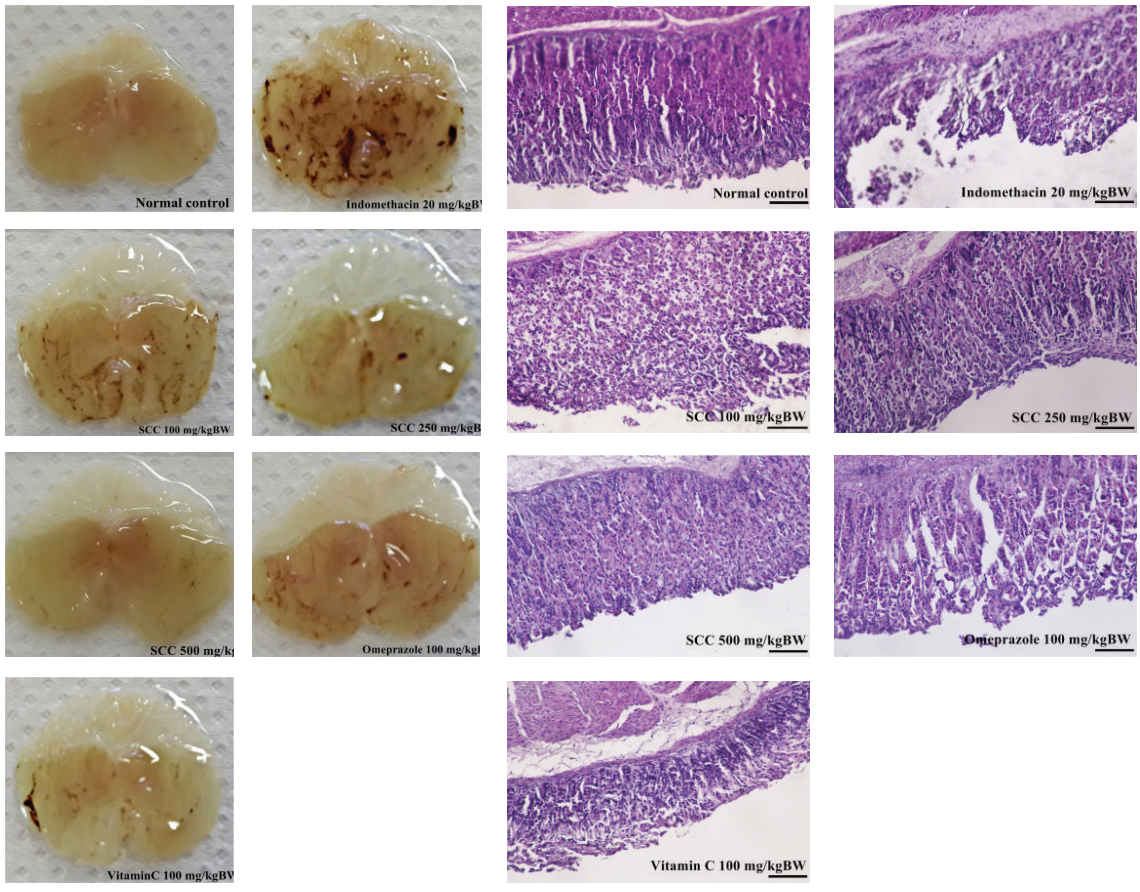

FIGURE 4: Photograph (a) and histology (b) of protective effect of SCC on gastric tissue after being induced with 20 mg/kgBW of indomethacin. Dark spot represented the ulcer area in gastric tissue and black bars represent size of tissue at 10 μm.

Table 1: Malondialdehyde (MDA), oxidized glutathione (GSSG), glutathione peroxidase (GPx), nitrite contents, gastric wall mucus, and plasma TNF-α in gastric tissue of SCC acute test group.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Mucus (μg Alcian blue/g wet stomach)	19.35 ± 0.94	15.78 ± 0.33^{a,c}	20.74 ± 0.75^{b}	21.13 ± 0.98^{b}	23.59 ± 0.55^{a,b,c}	21.31 ± 0.75^{a,b}	16.28 ± 0.64^{a,c}
MDA							
Plasma (nM/mL)	17.97 ± 2.69	23.99 ± 2.49^{a,c}	16.54 ± 5.53^{b}	16.45 ± 4.04^{b,c}	16.01 ± 3.19^{b,c}	19.06 ± 2.35^{a,b}	19.17 ± 2.55^{a,b}
Tissue (nM/mg tissue)	1.18 ± 0.11	1.55 ± 0.03^{a,c}	1.07 ± 0.29^{b}	1.03 ± 0.12^{b}	0.92 ± 0.04^{a,b}	1.04 ± 0.14^{b}	1.29 ± 0.06^{a,b,c}
GSSG (ng/mg Protein)	0.75 ± 0.01	1.26 ± 0.04^{a,c}	0.99 ± 0.04^{a,b}	0.87 ± 0.05^{a,b,c}	0.81 ± 0.01^{a,b,c}	0.98 ± 0.04^{a,b}	0.87 ± 0.01^{a,b,c}
GPx (U/g Protein)	2.41 ± 0.37	0.75 ± 0.19^{a,c}	1.17 ± 0.13^{a,b,c}	1.19 ± 0.16^{a,b,c}	2.59 ± 0.13^{b,c}	0.99 ± 0.05^{a,b}	0.89 ± 0.04^{a,b,c}
Nitric oxide (nM/mg Protein)	1.02 ± 0.31	2.39 ± 0.49^{a,c}	1.27 ± 0.311^{b,c}	1.07 ± 0.37^{b,c}	0.68 ± 0.23^{a,b,c}	1.54 ± 0.18^{a,b}	0.78 ± 0.11^{b,c}
Plasma TNF-α (pg/mL)	1.71 ± 0.04	2.09 ± 0.13^{a}	2.07 ± 0.09^{a,c}	1.92 ± 0.05^{a}	1.83 ± 0.03^{a,b}	1.89 ± 0.05^{a}	1.80 ± 0.06^{a,b}

$^{a}P < 0.05$ compared with normal mice group, $^{b}P < 0.05$ when compared with indomethacin treated group, and $^{c}P < 0.05$ when compared with omeprazole treated group.

with indomethacin, SCC 100–500 mg/kgBW, omeprazole, and vitamin C. The normal control group showed intact mucosal epithelium, submucosa, and muscularis mucosa. Indomethacin treated group had shown large erosion area in the mucosal epithelium with presence of crypts; ulcer pits and damage muscularis mucosal layer were observed. Treatment with SCC can protected the gastric tissue from the action of indomethacin damage cell. At the concentration of 100 and 250 mg/kgBW of SCC treated group the rupture at various portions of the mucosal epithelial layer was presented and the crypts in the mucosal glandular layer and slightly disrupted muscle layer similar results to omeprazole and vitamin C treatment group were showed. SCC at 500 mg/kgBW treated groups significantly reduced the damage of gastric cell and protected gastric cell as well as the normal control group.

3.3.3. Determination of Gastric Wall Mucus in Indomethacin-Induced Acute Gastric Ulceration. The alcian blue-binding capacity, which indicates the acidic mucopolysaccharide content of gastric mucin of normal control, indomethacin, SCC 100–500 mg/kgBW, omeprazole, and vitamin C 100 mg/kgBW treated groups were 19.35 ± 0.94, 15.78 ± 0.33, 20.74 ± 0.75, 21.13 ± 0.98, 23.59 ± 0.55, 21.31 ± 0.75, and 16.29 ± 0.64 μg alcian blue/g wet stomach, respectively. After mice were treated with PBS 1 h before giving indomethacin at 20 mg/kgBW mucus content was significantly decreased when compared to normal mice. In contrast, SCC 100–500 and omeprazole 100 mg/kgBW significantly increased mucus levels compared to indomethacin treated group which indicated the defensive system of SCC extract and omeprazole against indomethacin induced gastric ulcer. However, we found that vitamin C treated group showed no difference of mucus content compared to indomethacin treated alone group (Table 1).

3.3.4. Determination of SCC Inhibited Oxidative Damage on Indomethacin-Induced Acute Gastric Ulceration. The lipid peroxidation, GSSG, GPx, and NO were demonstrated in Table 1.

(1) Effect on Lipid Peroxidation. The MDA levels were increased markedly after being treated with 20 mg/kgBW of indomethacin in either plasma or gastric tissue when compared to normal mice ($P < 0.05$). In plasma treatment with SCC 100–500 mg/kgBW reduced the MDA levels compared with indomethacin treated group. The concentration of SCC at 250 and 500 mg/kgBW were significantly improved MDA levels better than omeprazole treated group ($P < 0.05$) and were not different from the MDA levels of untreated control mice.

MDA levels in gastric tissue were similar to plasma MDA levels in which pretreatment of PBS before 20 mg/kgBW of indomethacin significantly increased the MDA level ($P < 0.05$) when compared to normal mice while SCC treated groups decreased the levels of MDA in a dose dependent manner. SCC at 100–500 mg/kgBW significantly decreased MDA when compared with indomethacin treated group ($P < 0.05$) and maintained the level as well as normal mice. Omeprazole and vitamin C at 100 mg/kgBW also significantly reduced MDA levels compared to indomethacin treated group in gastric tissue.

(2) Effect on Oxidized Glutathione. Mice in indomethacin treated group showed a higher level of GSSG compared to normal mice. In contrast, the levels of GSSG were decreased in the SCC, omeprazole, and vitamin C compared to indomethacin treated group. The levels of GSSG in SCC at 500 mg/kgBW were reduced nearly to the GSSG level of normal mice.

(3) Effect on Glutathione Peroxidase (GPx). The GPx activity in gastric tissue was based on measurement of the oxidation of NADPH. The activities of the antioxidant enzymes GPx were significantly decreased in indomethacin treated mice compared to normal mice group. Treatment of SCC, omeprazole, and vitamin C before treatment with 20 mg/kgBW indomethacin increased the activities of GPx compared to indomethacin treated group. Treatment of SCC at 500 mg/kgBW protected the gastric mucosal against the loss of antioxidant enzyme activity, resulting in a significant increase in enzymatic GPx activities approximate to the normal control levels.

(4) Effect on Nitrite Contents. The nitrite content of indomethacin treated group was significantly increased compared to the normal control group ($P < 0.05$), while the nitrite content of the SCC, omeprazole, and vitamin C was significantly lower than that in the indomethacin treated group.

3.3.5. The Effect of SCC on Inflammation Parameter

(1) Plasma TNF-α. Plasma TNF-α level of indomethacin treated group was at 2.09 ± 0.13 pg/mL and was significantly increased when compared to normal mice ($P < 0.05$). Pretreatment of SCC at concentration of 500 mg/kgBW or vitamin C showed significantly reduced TNF-α levels compared to indomethacin treated group ($P < 0.05$), while omeprazole treated group was not significantly different compared to indomethacin treated group (Table 1).

(2) The Expression of COX-1, COX-2, and iNOS. Figure 5 showed the expression of COX-1, COX-2, and iNOS on SCC protective effect on indomethacin-induced gastric ulcer. The expressing of COX-1 was reduced after 20 mg/kgBW indomethacin administration and COX-2 was increased in the same group. The iNOS protein levels in indomethacin treated mice indicated upregulated iNOS gene expression. However, treatment with SCC significantly reduced the expression of this enzyme ($P < 0.05$). Thus, SCC, omeprazole, and vitamin C which reduced the expression of iNOS enzyme level led to the decrease of the NO level which further prevented the acute phase inflammation and regulation of the severity of gastric ulcer.

4. Discussion

Syzygium cumini (L.) Skeels leaves contain various medicinal compounds and were used for treatment in various pharmaceutical preparations. It is well-known that natural phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health benefit effects. The natural phenolic compounds also serve in plant as defense mechanisms to counteract ROS in order to survive and prevent molecular damage and damage by microorganisms, insect, and herbivores [19]. Total phenolic compounds, including tannins and flavonoids, have been reported to have multiple biological properties to possess general antimicrobial and antioxidant activity [20, 21]. The strong antioxidant activity of the SCC may be attributed to the phenolic compound and flavonoid content which were correlated with r^2 value at 0.93. In this study, SCC scavenge free radical $ABTS^{\bullet+}$, hydroxyl, superoxide anion, and nitric oxide radical *in vitro* indicated that the SCC may help to arrest the chain of reactions initiated by excess generation of $OH^{\bullet}$, $O_2^{\bullet}$, and $NO^{\bullet}$ that similarly reported the effectiveness in human health [21]. SCC also showed no pathological side effect on tissues damage in both liver and kidney tissue.

Indomethacin possesses the highest ulcerogenic potential to humans among the commonly used NSAIDs. The gastric damage induced by indomethacin in mice can be attributed to their ability to induce the reactive oxygen metabolites. The notion that SCC showed powerful *in vitro* antioxidant property encouraged us to investigate their possible protective effect against indomethacin NSAIDs induced gastric ulcer. The induction of gastric ulceration by indomethacin in acute model was associated with gastric lipid peroxidation and inflammatory reaction to produced ROS which leading to severe ulceration on gastric tissue and were demonstrated on UI values, photograph, and pathology of gastric mucosa of this work. The reduction of gastric ulcer area found in SCC treated group according to their ROS scavenge resulted in increasing inhibitory percent.

The gastric mucosal barrier is a complex system made up of submucosal epithelial and mucus elements [22]. The mucus gel layer is a thick organized layer adherent to the epithelium and plays an important role in protection of the epithelium against acid, pepsin, and mechanical damage [23, 24]. The stimulation of production of gastric mucin, PGE_2, and bicarbonate and the decrease of acid output help to maintain mucosal integrity. An increase in mucus production usually assists the healing process by protecting the ulcer crater against the endogenous aggressors like stomach secretions and oxidants as well as against exogenous damaging agents such as NSAIDs. These results revealed that stomach ulceration in indomethacin treated group resulted in decreased mucin secretion. This might reduce the ability of the mucosal membrane to protect the mucosa from physical damage and back diffusion of hydrogen ions. Apparently, the antioxidative property of the SCC might be contributing in protecting the oxidative damage to gastric mucosa.

Tissue damage is always associated with excess generation of ROS leading to oxidative stress. ROS mediated lipid peroxidation is one of the principle causes of gastrointestinal lesion induced by indomethacin [25]. The free radicals, in particular hydroxyl radical, begin the peroxidation of cell membrane, causing the liberation of arachidonic acid and peroxyl lipid free radical [26]. The peroxyl radical promotes additional lipid peroxidation, removing hydrogen from fatty acid and originating a chain reaction in which the final product is the MDA [27].

SCC was corroborated by preventing the indomethacin-induced lipid peroxidation. The ROS-mediated degradation of cell membrane results in the formation of lipid peroxides and initiates a variety of deleterious sequence, including mucosal lesions and depletion of mucus layer, alterations that were confirmed in this work in indomethacin treated group. The gastroprotection of SCC depends also on replenishing the increased GSSG. The endogenous defense molecule

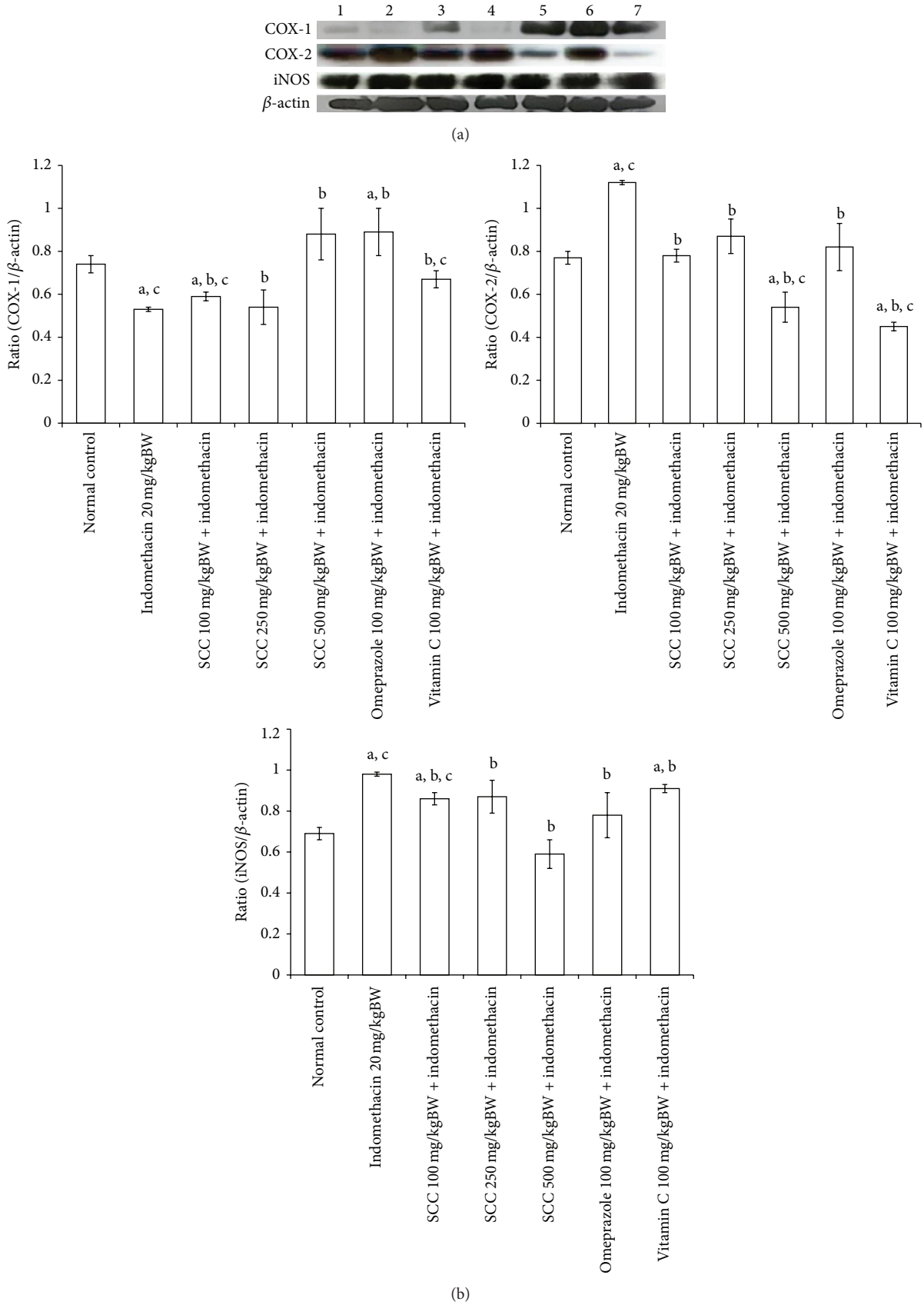

Figure 5: COX-1, COX-2, and iNOS expression in gastric tissue of acute study. Western blot was represented, showing the ratio of COX-1, COX-2, and iNOS compared to β-actin band ((a) and (b)). Analysis represents mean ± S.E.M. a: $P < 0.05$ compared with normal mice, b: $P < 0.05$ compared with indomethacin treated group, and c: $P < 0.05$ compared with omeprazole treated group.

maintains the normal redox potential and counteracts free radical-or-toxic induced damage including superoxide dismutase (SOD) and glutathione peroxidase (GPx) [28, 29]. Indeed, our work showed that SCC leveled up GPx activity above the normal level and hence improves the endogenous defense system, which was demolished by the indomethacin direct inhibitory effect on peroxidases [30] and hydrogen peroxide overproduction by the gastric mucosa [31]. The high activity may explain the prevention of lipid peroxidation by SCC, especially in the presence of normal level of its product, GSSG.

Nitric oxide (NO) is a crucial mediator of gastrointestinal mucosal defense but it also contributes to mucosal damage [32]. This can be illustrated by the ability of different nitric oxide concentration to produce completely opposite effects in the same tissue [33]. There is evidence that low doses of NO releasing substances protect against NSAID induced gastropathy and increase the healing rate of gastric ulcers. However, high doses of these substances could induce extensive hemorrhagic mucosal damage [34]. NO is produced by NOsynthase (NOS) in various types of cells, including endothelial cells, neurons, neutrophils, macrophages, and brush cells of the gastric surface epithelium [35]. There is growing evidence that the major source of ROS is from the activated neutrophils [36] and neutrophils play a vital role in the development of gastric damage by their aggregation and release of tissue disturbing substance such as oxygen free radicals and proteases [37]. Therefore, the NO levels of indomethacin treated group in acute study were increased due to the directly stimulated NO from neutrophils and macrophages. Thus, SCC reduced the nitrite concentration compared to indomethacin treated group by their scavenger property.

TNF-α seems to be one of the key factors for several forms of mucosal gastric lesions including NSAIDs. The plasma levels of TNF-α have been observed to increase markedly after administration of NSAIDs. Also, TNF-α plays a critical role in NSAIDs induced gastric injury by modulating neutrophil infiltration [38] and induces leucocytes adherence after indomethacin administration [18]. Thus, SCC inhibited the increase production of proinflammatory cytokines, TNF-α, through its antioxidant effects.

NSAIDs inhibit prostaglandin (PG) production by inhibiting COX. Two isoforms of COX enzyme are known to be involved in PG synthesis. COX-1 is constitutively expressed and generates PG involved in gastrointestinal protection and platelet function, while at site of inflammation, COX-2 is induced to generate PG which mediates inflammation and pain [39]. The NSAIDs like indomethacin exert both their therapeutic and toxic effects mainly through inhibiting cyclooxygenases and decreasing the levels of circulating PGE_2 at gastric mucosa causing gastric ulcerations and also exacerbating preexisting gastric ulcers in rodent and humans [40]. Indomethacin has a weak selectivity for COX-1 and we can argue that the inhibition of COX-2 by intravenously administered indomethacin was not enough, thereby resulting in the development of gastric lesions. The present report shows a reduction of COX-1 was expressed after 20 mg/kgBW indomethacin administration and COX-2 was increased in the same group. This finding can explain that the high doses of indomethacin was induced gastric lesions than to inhibited COX-2 expression on the gastric mucosa, because higher concentrations were required for inducing the direct cytotoxic effect of NSAIDs than were required for inhibiting PG synthesis [41]. Therefore, the compound which is selective for COX-2 should theoretically provide efficacy comparable to traditional nonselective NSAIDs without inducing the gastrointestinal injury or platelet dysfunction as in our studies; SCC stimulated the expression of COX-1 and inhibited the expression of COX-2 by dose dependent manner.

Omeprazole is one of drugs used for the prevention of gastrointestinal side effects of NSAIDs. In previous study, omeprazole provided more effective protection than placebo in the healing of both gastric and duodenal mucosa damage [42, 43]. The protective effects of pretreatment with omeprazole are compared to SCC in indomethacin treated mice. Omeprazole had significant protection effects compared to indomethacin treated group but the prevention effect of omeprazole was less than SCC, may be due to the ROS scavenging action of natural compounds. Similar to our study, it has been reported that omeprazole decreased the production of gastric ulcers by more than 80% [44]. However, in terms of drug usage, combination of omeprazole and SCC could be rise up the prevention processes through breaking ROS and proton pump action in gastric ulcer and reduced the cost on treatment processes. Vitamin C was used as a reference antioxidant compound to support the antioxidant action of SCC on indomethacin-induced gastric ulcer. However, the high dose of vitamin C (100 mg/kgBW) in our study did not show the good protection in indomethacin-induced acute ulceration when compared to SCC and omeprazole treated groups.

5. Conclusions

SCC has shown an excellent in *in vitro* and *in vivo* antioxidant and ROS scavenging activity and has been established as a natural antioxidant. SCC has shown the strong action in antioxidant and anti-inflammatory activities. Furthermore, SCC is action as anti-inflammatory compounds by breaking of NO, TNF-α in inflammatory process, reduced the inhibition of COX-1 and drop downed the expression of COX-2, resulting in the reduction of severe damage of gastric cells from indomethacin action. From this powerful action of SCC, we can summarize that, SCC can be used as anti-inflammation and antigastric ulcer compound without toxicity.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This work was supported by Walailak University Fund and the authors express thanks to the School of Allied Health Sciences and Public Health, Walailak University, for providing all the facilities to conduct this work.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2015, Article ID 132190, 10 pages
http://dx.doi.org/10.1155/2015/132190

Research Article

Molecular Features and Methylation Status in Early Onset (≤40 Years) Colorectal Cancer: A Population Based, Case-Control Study

Giulia Magnani,[1] Daniela Furlan,[2] Nora Sahnane,[2] Luca Reggiani Bonetti,[3] Federica Domati,[1] and Monica Pedroni[1]

[1]*Dipartimento di Medicina Diagnostica, Clinica e Sanità Pubblica, Università di Modena e Reggio Emilia, 41124 Modena, Italy*
[2]*Dipartimento di Chirurgia e Scienze Morfologiche, Università dell'Insubria, 21100 Varese, Italy*
[3]*Divisione di Anatomia Patologica, Policlinico di Modena, 41124 Modena, Italy*

Correspondence should be addressed to Monica Pedroni; monica.pedroni@unimore.it

Received 20 January 2015; Accepted 2 April 2015

Academic Editor: Miroslav Zavoral

Colorectal cancer is usually considered a disease of the elderly. However, a small fraction of patients develops colorectal cancer earlier. The aim of our study was to define the frequency of known hereditary colorectal syndromes and to characterise genetic and epigenetic features of early nonhereditary tumors. Thirty-three patients ≤40 years with diagnosis of colorectal cancer and 41 patients with disease at >60 years of age were investigated for MSI, Mismatch Repair proteins expression, *KRAS* and *BRAF* mutations, hypermethylation, and LINE-1 hypomethylation. Detection of germline mutations was performed in Mismatch Repair, *APC* and *MUTYH* genes. Early onset colorectal cancer showed a high incidence of hereditary forms (18%). *KRAS* mutations were detected in 36% of early nonhereditary tumors. Early onset colorectal cancer disclosed an average number of methylated genes significantly lower when compared to the controls ($p = 0.02$). Finally both of the two groups were highly methylated in *ESR1*, *GATA5*, and *WT1* genes and were similar for LINE-1 hypomethylation. The genetic make-up of carcinomas differs from young to elderly patients. Early onset tumors showed more frequently a constitutional defective of Mismatch Repair System and a minor number of methylated genes. Hypermethylation of *ESR1*, *GATA5*, and *WT1* genes suggests possible markers in the earlier diagnosis of colorectal tumorigenesis.

1. Introduction

Colorectal cancer (CRC) is the second most common cancer in both men and women in Western Europe, North America, Australia/New Zealand, and Japan, whereas it remains less frequent in Africa and Asia [1]. Colorectal cancer is usually considered a disease of the elderly in both sexes; data from Cancer Registries indicate that age-specific cancer incidence rises sharply after the age of 50–55 years and that mean age of affected individuals is around 70 years [2]. However, in a small fraction of patients (2-3% of all affected individuals), colorectal malignancies may develop earlier [3].

A review of the Surveillance Epidemiology and End Results (SEER) Program data from 2005 to 2009 provides more detailed information regarding colorectal cancer in younger patients suggesting that the incidence in younger patients is increasing along time (ages <20 to 54 years) [4], whereas other data would show a relative stability of the rates [5]. Part of these discrepancies can be attributed to the different age-limits proposed for the definition of early onset (or "juvenile") tumors, which have been set at age 40, 45, or 50 depending on the authors and purposes of the studies [6, 7]. Although there is no clear age cut-off defined, the majority of studies classify patients diagnosed with cancer at age <40 years as "early onset" (or "young") [8]. The reasons whereby some individuals develop colorectal cancer at an unusual age are poorly understood. Therefore, the etiology and biological characterisation of the majority of early onset colorectal neoplasms remain poorly defined.

From a molecular point of view, early onset tumors represent a heterogeneous group of diseases, including known hereditary syndromes, familial cases, and apparently sporadic colorectal cancer. Hereditary diseases explain only part of early onset colorectal cancer [9] and mainly include Lynch syndrome [7, 10–12], Familial Adenomatous Polyposis (FAP) [13], Peutz-Jeghers syndrome [14], Cowden syndrome/Bannayan [15], and *MUTYH*-associated polyposis (MAP) [13, 16].

Recent studies have suggested that the clinic-pathologic features related to early onset colorectal cancer [7] are more likely to be present at advanced stages, to be poorly differentiated, and to be located in the distal colon and in the rectum, although marked differences have never been reported. Morphologically, early onset CRCs more frequently display adverse histological features, such as signet ring cell differentiation and perineural and venous invasion [9]. Investigation of somatic profiles showed an increasing number of tumors with *KRAS*, *BRAF*, and *PIK3CA* oncogenes mutations related to advanced age. Mutations in the tumor suppressor genes *TP53* and *PTEN* were more frequent in the early onset group [17]. Other findings showed that young patients had significantly more chromosomal aberrations in their tumors than patients aged >70 years and microsatellite stable (MSS) colorectal cancer [7, 17, 18]. Recently, LINE-1 hypomethylation has been reported to be a distinct feature of MSS young age colorectal cancers (≤45 years), suggesting that the genomic hypomethylation may represent a possible pathway of early onset colorectal carcinogenesis [19]. By contrast, very few data are available about the frequency of CpG island methylator phenotype (CIMP) in early onset CRCs [19].

The knowledge about this clinically and genetically heterogeneous group of colorectal cancers remains limited and further studies are essential. To address this issue, we conducted a population based case-control study on CRCs diagnosed before the age of 40 years in order to define the frequency of known hereditary CRC syndromes in this subset of patients and to better characterize genetic and epigenetic features of early nonhereditary tumors compared with sporadic late onset CRCs.

2. Materials and Methods

2.1. Patients. The patients described in this study were recruited through the specialized Colorectal Cancer Registry of Modena in the period 1984–2008. In the 25 years of registration, we recruited 38 patients affected by adenocarcinoma in the colon-rectum diagnosed ≤40 years, which represented about 1% of the overall adenocarcinomas diagnosed (4,692 cases). We collected the biological material (blood sample, normal colorectal mucosa, and tumoral tissue) of 33 cases out of 38 and investigated the family history relative to the cases, including at least first-degree or second-degree relatives.

In order to compare some molecular features—including methylation of DNA—of early onset colorectal cancer with maturity onset disease, we selected a specific control group composed of 41 patients with late onset colorectal cancer (age-range: 61–85 years) that were matched to the study group for the following characteristics: sex, tumor location, and stage. Family cancer history was not present in late onset group. Tumors were classified as low-grade and high-grade adenocarcinoma [20] and staged with the Dukes' classification.

The study was approved by the local ethics committee. All individuals, or first-degree relative in case of death of the index case, gave their written consent for blood samples and tissue specimen analysis.

2.2. DNA Extraction. Genomic DNA from each patient was extracted by formalin-fixed, paraffin-embedded normal and tumor tissues. Serial sections from paraffin-embedded matched normal and neoplastic primary tissue were stained with Hematoxylin-eosin; representative normal and tumor regions were identified by microscopic examination. Areas of tumor tissue with more than 80% of malignant cells were selected in all cases, as previously described in detail [21]. Constitutional DNA from peripheral blood was obtained using QIAamp DNA Mini kit (Qiagen), according to the manufacturer's instructions.

2.3. MSI Analysis. MSI status of all tumors was evaluated using four fluorescent-labeled mononucleotide markers: BAT25, BAT26, NR24, and CAT25. These quasimonomorphic markers were selected after in-depth review of the literature for their very high sensitivity and specificity in identifying Mismatch Repair-deficient tumors [22–24]. The resulting panel composed of four markers reduced the time and cost involved in MSI testing. Other authors as Xicola and Deschoolmeester suggested a MSI analysis shortcut [23, 24]. Using this mononucleotide markers panel, a tumor was defined as MSI(+) when showing instability with at least three markers. MSI analysis was performed as described previously [21].

2.4. Immunohistochemical Analysis of MMR Proteins. Immunohistochemical (IHC) evaluation of MMR proteins expression was carried out on paraffin-embedded tissue sections of all tumors. The following mouse monoclonal antibodies were used: anti-MLH1, anti-MSH2 (PharMingen, San Diego, CA), and anti-MSH6 Transduction Laboratories, BD Biosciences, Brazil. For PMS2 protein, a rabbit monoclonal antibody was used (Ventana Roche Diagnostic, Italy). Immunostaining was executed by the avidin-biotin peroxidase technique; diaminobenzidine was used as a chromogen. Staining was carried out in NEX-ES Automatic Staining System, after counterstaining with Hematoxylin. Normal tissue and stromal cells or lymphocytes adjacent to the respective tumor were used as internal positive controls. Loss of MMR proteins expression was defined as complete absence of nuclear staining in tumor cells (but maintained in normal epithelial and stromal cells).

2.5. Analysis of Germline Mutations in MMR, APC, and MUTYH Genes. Cases showing MSI and lack of expression of one of MMR proteins in tumors were investigated to detect germline mutations in *MMR* genes. Patients with clinical

features of Familial Adenomatous Polyposis were assessed for *APC* gene and all young patients were screened for the presence of *MUTYH* gene mutations. Analysis of germline mutations was performed by direct sequencing of the PCR products obtained using the Dye Terminator Cycle Sequencing Kit (CEQ DTCS Kit, Beckman Coulter) and reactions were run on a CEQ 8000 capillary sequencer (Beckman Coulter). To exclude the possibility of large genomic rearrangements in *MMR* genes, we used Multiplex Ligation-Dependent Probe Amplification (MLPA) procedure by SALSA P003-B2 kit (MRC-Holland, Amsterdam, Netherlands) and to confirm results SALSA P248-A2 kit (MRC-Holland). The entire open reading frame of *APC* gene was also analysed for the presence of deletions or rearrangements by using the SALSA P043 kit (MRC-Holland). Pathogenic mutations were detected twice and confirmed in a second blood sample of the patient.

2.6. Somatic BRAF and KRAS Mutations Analysis. All 74 tumors were analysed for *KRAS* and *BRAF* activating mutations. In *KRAS*, the more frequently mutated codons 12, 13, and 61 were analysed [25]. In *BRAF* gene, we amplified exon 15 which includes the hot spot for mutation codon 600 (V600E). Mutation analysis was performed by direct sequencing with the use of the standard protocol and running on Beckman Coulter CEQ 8000 instrument.

2.7. Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) Analysis. MS-MLPA analysis was performed on all the 74 tumors (33 early onset CRC and 41 control cases) using the ME001 MS-MLPA Tumor Suppressor-1 Kit, the ME002 MS-MLPA Tumor Suppressor-2 Kit, and the ME011 MS-MLPA Mismatch Repair Genes Probemix Kit (MRC-Holland, Amsterdam, Netherlands). Using these three kits, a total of 38 tumor suppressor genes were analysed for aberrant promoter methylation. All these genes are frequently silenced by methylation in tumors of different sites, and they frequently harbour genetic alterations during tumorigenesis. Methylation-specific MLPA (MS-MLPA) is a semiquantitative method for methylation profiling. MS-MLPA is a variant of the MLPA technique in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme [26]. Probe sequences, gene loci, and chromosome locations can be found at http://www.mlpa.com. The experimental procedure was carried out according to the manufacturer's instructions. Reaction products were separated on an automated sequencer (ABI 310 capillary) and visualised with Genemapper analysis v.4.0 (Applied Biosystems). Values corresponding to peak size (base pairs) and peak height were used for further data processing by Coffalyser V7 software (MRC-Holland). All MS-MLPA reactions were performed at least two times. The methylation profile of each sample was assessed according to MRC-Holland instructions.

Aberrant methylation was scored as a categorical variable using a specific Methylation Ratio (MR) for each gene corresponding to the highest level of accuracy of the test, according to previously reported [27].

2.8. LINE-1 PCR and Pyrosequencing. The methylation status of LINE-1 was evaluated by bisulfite-PCR and pyrosequencing [28] in all tumors and in twenty-five samples of normal colonic mucosa. Thirteen of these samples were derived from normal tissue at the resection margins of 13 patients with sporadic CRCs. The remaining normal specimens were obtained from 12 individuals who had undergone surgery for ischemic colorectal disease or for diverticulitis without a personal history of colorectal cancer. Bisulfite treatment of genomic DNA converts all unmethylated cytosines into thymine while methylated cytosines remain unchanged. All the cytosine residues unconverted in the sequence represent methylated cytosines in the genome. In this method, 1.5 mg of DNA was denatured in 50 mL of 0.2 M NaOH for 10 min at 37°C. Then, 30 mL of freshly prepared 10 mM hydroquinone and 520 mL of 3 M sodium bisulfite at pH 5.0 were added and mixed. The samples were incubated at 50°C for 16 h. The bisulfite-treated DNA was purified using Wizard DNA Clean-Up System (Promega).

LINE-1 assay was designed toward a consensus LINE-1 sequence (GenBank accession number X58075) and allowed to quantify the percentage of 5-methylated cytosines (%5mC) in five consecutive CpG sites. PCR was performed in a 50 μL reaction volume that included 2 pmol of forward primer 5′-GAGTTAGGTGTGGGATATAGT-3′, 2 pmol of reverse biotinylated primer 5′-CAAAAAATCAAAAAA-TTCCCTTCCC-3′, 5 μL of bisulfite-treated genomic DNA 1.25 units of GoTaq DNA polymerase, 1X GoTaq Flexi Buffer (Promega, Madison, WI, USA), and 200 μM dNTPs. Thermal cycling conditions were 3 min at 95°C, 45 cycles at 95°C/25 s, 50°C/25 s, and 72°C/25 s, followed by a final extension at 72°C for 5 min. Pyrosequencing was performed on PCR product with bound LINE-1 sequencing primer 5′-GGTGTGGGATATAGTT-3′, according to the protocol reported above. Fully methylated DNA (CpGenome Universal Methylated DNA, Millipore, Billerica, MA, USA) and unmethylated DNA (CpGenome Universal Unmethylated DNA, Millipore, Billerica, MA, USA) were used as positive and negative controls for optimizing the assay.

2.9. Statistical Analysis. Univariate comparisons of continuous data were carried out using Student's t-test and discrete variables were compared with χ^2 test or Fisher's exact test. The association between discrete outcome and continuous predictor was evaluated with a logistic regression model. All comparisons were two-sided and a p value < 0.05 was considered to be significant.

3. Results

3.1. Patients Features. We recruited a total of 38 patients with adenocarcinoma in the colon-rectum diagnosed before the age of 40 years, but the biological material was available for 33 patients. Clinicopathological features of the 33 colorectal adenocarcinomas and patients are shown in Table 1. The mean age of disease onset was 35 years. We observed that the male gender was approximately 3 times more frequent than female (76% versus 24%), tumor location was preferentially the left

Table 1: Clinicopathological features of 33 colorectal adenocarcinomas (and patients) developed ≤40 years (1984–2008) compared to patients with cancer onset >60 years.

	Technical details	Patients with onset ≤40 years, $N = 33$ (%)	Patients with onset >60 years, $N = 41$ (%)
Age of onset of disease	Min	11	61
	Max	40	85
	Mean	35	73
Sex	Female	8 (24)	11 (27)
	Male	25 (76)	30 (73)
Tumor location in the large bowel	Right colon	7 (21)	12 (29)
	Left colon	17 (52)	19 (47)
	Rectum	9 (27)	10 (24)
Stage (Dukes)	A	5 (15)	7 (17)
	B	7 (21)	9 (22)
	C	12 (37)	14 (34)
	D	9 (27)	11 (27)
Tumor differentiation	Low-grade	19 (58)	31 (76)
	High-grade	8 (24)	8 (19)
	Mucinous	6 (18)	2 (5)

colon (52%), and C and D stages of Dukes (37% and 27%, resp.) were more frequently represented. Fifty-eight percent of early onset tumors were well or moderately differentiated. Family history of colorectal cancer was present in 8 (24%) patients: 2 with Bethesda criteria (patients with a first-degree relative affected by colorectal cancer), 5 who fulfilled the Amsterdam II criteria, and 1 with Familial Adenomatous Polyposis. Clinical features of Cowden/Bannayan [29] and Peutz-Jeghers syndromes [30] were not identified in any patients.

3.2. MMR Deficiency and Somatic Mutations of KRAS and BRAF Genes. MMR deficiency was evaluated by both immunohistochemistry and MSI analysis in all 74 colorectal cancers. MMR deficiency was defined as loss of protein expression in any of the MMR proteins and/or having a MSI tumor. Seven out of 33 (21%) early onset tumors showed MSI and loss of expression of MMR proteins (4 for MLH1/PMS2 and 3 for MSH2/MSH6). By family history, 5 patients (≤40 years) with MMR deficient tumors fulfilled clinical features of Lynch syndrome and 2 the Bethesda Criteria. Four out of 41 (10%) tumors in the control group (4 for MLH1/PMS2) were MMR deficient. These 4 tumors diagnosed over the age of 60 years showed somatic *MLH1* hypermethylation and V600E mutation in *BRAF* gene. No V600E mutations were detected in MSS tumors with clinical onset at advanced age or in early onset colorectal cancers. We found somatic *MSH2* methylation in only one case (patient with germinal *EPCAM* deletion) among 7 young patients showing MSI and loss of MSH2/MSH6 protein expression. *KRAS* mutations were found in 10 early onset tumors and in 4 cases of the control group. Thus, tumors of juvenile cases showed more often somatic *KRAS* mutations (30% versus 10%). Molecular results in the investigated patients are summarized in Table 2.

3.3. Germline MMR, APC, and MUTYH Mutations. We identified 6 cases with sequence variants in either *MLH1* (3 cases), *MSH2* (1 case), *EPCAM* (1 case), or *APC* (1 case) (Table 3). For *MLH1*, we detected one insertion mutation (p.Arg497ProfsX6) and two missense mutations (p.Leu749Pro and p.Glu663Asp). These two missense variants were classified as Class 5 mutations (>99% likelihood of pathogenicity) by Plon et al. [31]. For *MSH2*, we found one nonsense mutation (p.Phe294X). Moreover, we identified a large deletion of exons 8 and 9 in *EPCAM* gene, without involvement of *MSH2* promoter, using the P003-B2 MLPA kit. This finding was confirmed by the P072-B1 MLPA kit (MRC-Holland). Only one patient was affected by Familial Adenomatous Polyposis and showed an insertion mutation in *APC* gene at exon 15 (p.Arg924SerfsX16). Germline analysis of *MUTYH* gene was carried out in the whole group of young patients, but no monoallelic or biallelic alteration was detected.

3.4. Gene-Specific DNA Methylation. MS-MLPA assay was employed to evaluate the hypermethylation profiles relative to tumors from early onset patients ($n = 33$) and control group ($n = 41$). The average number of methylated genes was significantly higher in the control group compared with the young group (5.025 *versus* 3.3, resp.) ($p = 0.02$, Figure 1(a)). By contrast, no significant differences were observed between young and hereditary/suspect of hereditary cases (Figure 1(b)).

In the control group, we observed two main tumor sets on the basis of the degree of methylation. The first group consisted of 7 CRCs (17% of cases) showing high levels of gene methylation, involving a mean percentage of 28% of the promoters examined (ranging from 24% to 37%). The second group included the remaining 34 CRCs showing absent or low level methylation involving a mean percentage of 9% of the genes analysed (ranging from 0% to 20%) (Figure 2).

Table 2: Molecular features of colorectal carcinomas in cases and in the control group.

Technical detail	Patients with onset ≤40 years $N = 33$		Control group >60 years, $N = 41$ (%)
	Nonhereditary cases, $N = 25$ (%)	Hereditary/suspect of hereditary cases, $N = 8$ (%)	
MMR alterations			
MSI and no expression MMR proteins	0	7 (88)	4 (10)
Methylation			
MLH1 or *MSH2* promoter hypermethylation	0	1 (13)	4 (10)
Somatic mutations			
KRAS mutation	9 (36)	1 (13)	4 (10)
BRAF mutation	0	0	4 (10)
Germline mutations			
MMR	0	5 (63)	0
APC	0	1 (13)	0
MUTYH	0	0	0

Table 3: Constitutional mutations in early onset colorectal cancer.

	Cases	Gene	Mutation
Lynch syndrome	5	*MLH1* (3)	c.2246T>C; p.Leu749Pro
			c.1489dupC; p.Arg497ProfsX6
			c.1989G>T; p.Glu663Asp
		MSH2 (1)	c.881_882delTT; p.Phe294X
		EPCAM (1)	Del ex 8-9 and seq in 3′ to +3 kb
Familial Adenomatous Polyposis	1	*APC*	c.2771_2772insT; p. Arg924SerfsX16

The seven CRCs exhibiting extensive gene methylation included all the cases showing MSI and *BRAF* mutation and *MLH1* methylation.

On the other hand, in the group of early onset tumors, we did not detect any case with extensive gene methylation. No methylation for the *MMR* genes was found in these tumors, with the exception of the case with *EPCAM* gene mutation (A34), which was methylated in the *MSH2* gene. Evaluating the hypermethylation frequency in single genes, no significant differences were observed comparing early onset patients and control group. By contrast, a very high frequency of methylation was detected in *ESR1*, *GATA5*, and *WT1* genes in both groups of the familiarity (Figure 3).

3.5. LINE-1 Hypomethylation. We used the quantitative bisulfite pyrosequencing to determine the methylation status of LINE-1 repetitive sequences in all CRCs compared to twenty-five samples of normal colonic mucosa. In normal samples, LINE-1 methylation levels were high (average 60.78% ± 0.4%) and very similar to those commonly observed in peripheral blood cells from normal individuals [32]. By contrast, LINE-1 methylation levels in CRCs were significantly lower than in normal samples (mean 55.4% ± 0.86; $p < 0.024$).

Mean LINE-1 methylation levels in the three study groups of colorectal cancers were early onset, 54.3%; late onset, 55.9%; Lynch syndrome, 59.0% (Figure 4). The difference of LINE-1 hypomethylation in early onset colorectal cancer was not significant when compared to late onset ones. Interestingly, in the Lynch syndrome tumors LINE-1 methylation levels were higher than early onset and late onset groups and similar in the mean percentage of normal mucosa. However, this difference did not reach statistical significance because of the small number of Lynch tumors.

4. Discussion

In this study we have assessed the clinicopathological, molecular, and familial features of 33 early onset colorectal cancers (≤40 years). We showed that the frequency of known hereditary colorectal cancer syndromes in this population was 18%. This cohort disclosed a molecular profile of *MMR* deficiency characterised by germline mutations in *MLH1*, *MSH2*, and *EPCAM* genes, thus confirming that Lynch syndrome is the most frequent cause of hereditary colorectal cancer in young patients [12, 19].

Previous studies have revealed that colorectal cancer diagnosed at early ages had a high probability of showing *MMR* deficiency, ranging from 26% to 73% [11]. The reason for this wide range could be explained by the different age thresholds (from 24 to 50 years) and by diverse panels of

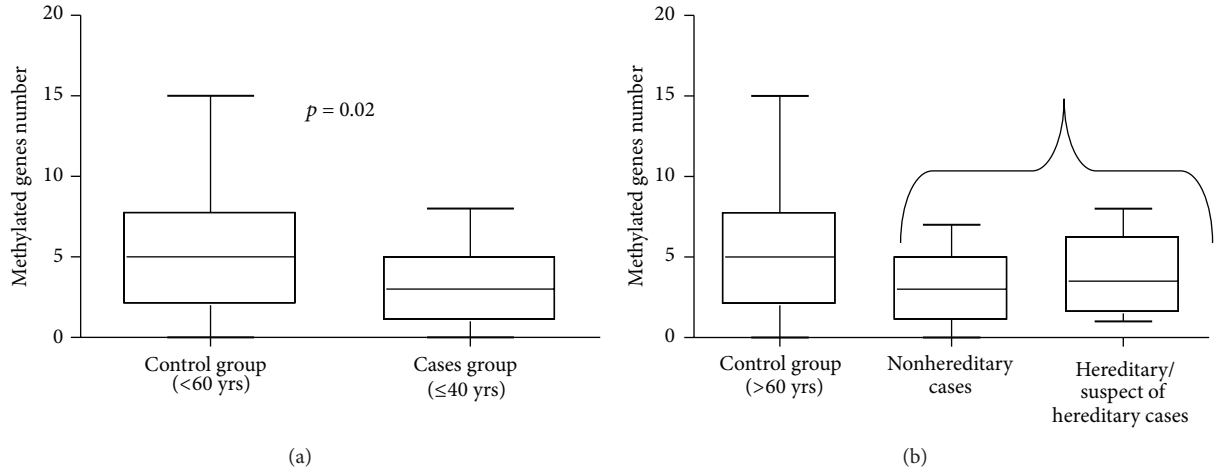

Figure 1: Results of methylation status in young group and in elderly group. In (a), we observed a significant difference in methylation pattern between patients under 40 yrs and patients over 60 yrs. The mean number of methylated genes in the control group is higher than average number in the cases group. In (b), we observe no statistical difference between control, young, and hereditary cases, but the difference remained significant between patients under 40 yrs and patients over 60 yrs.

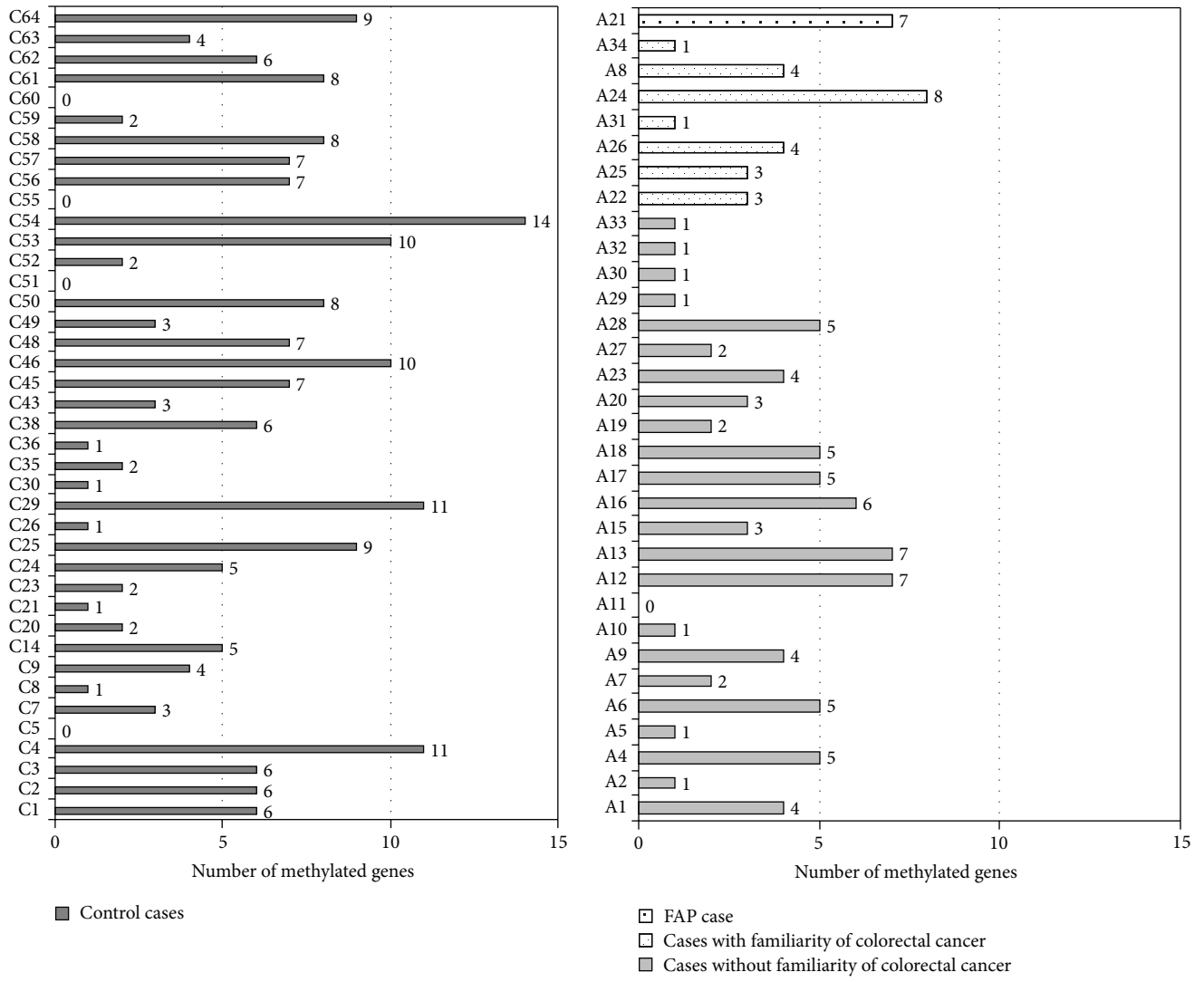

Figure 2: Number of methylated genes in control cases and in early onset colorectal cancer.

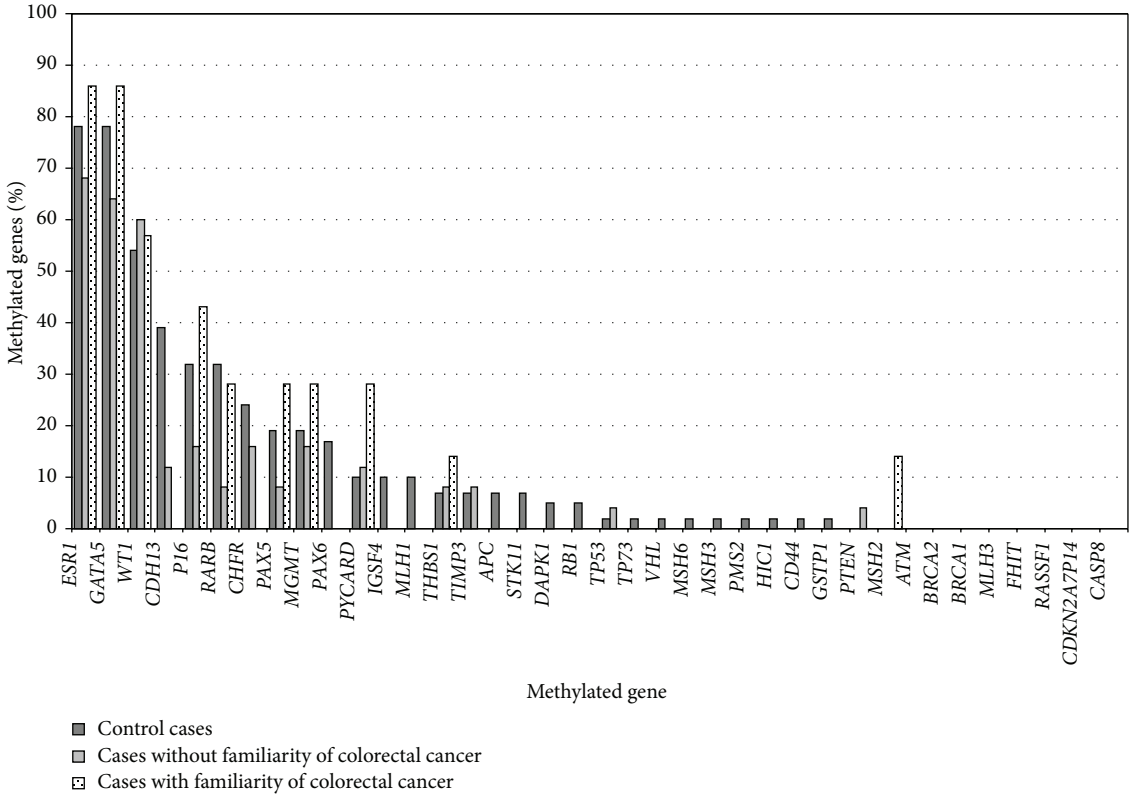

Figure 3: Hypermethylation frequency in single genes.

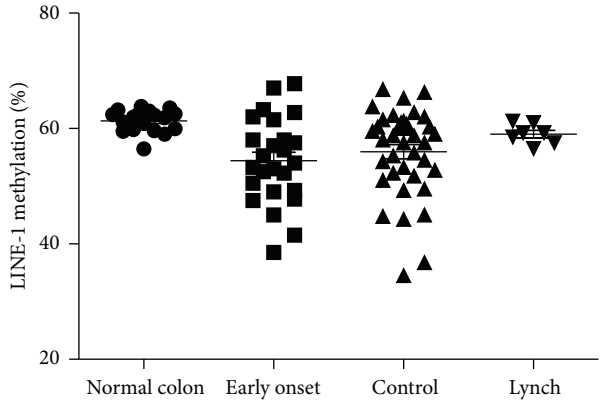

Figure 4: LINE-1 hypomethylation in early and late onset patients.

proteins analysed by IHC (usually restricted to MLH1 and MSH2). Moreover, the majority of studies have analysed either Lynch syndrome or sporadic colorectal cancer and it is known that the rate of MSI is much lower in the latter. Our study, based on a population based approach of colorectal cancer developed ≤40 years recruited from the specialised Colorectal Cancer Registry of Modena, showed that the frequency of MMR deficiency was 21%. Although we identified 7 tumors with MMR deficiency, we detected only five patients with pathogenetic germline mutations in *MMR* genes. Possible causes for the lack of identified constitutional mutations could be low sensitivity of analytical methods or genetic events [33] that affect both alleles of a *MMR* gene. We did not identify any carrier of *MSH6* germline mutations. Recent studies have shown that the average age of colorectal cancer onset in *MSH6* mutation carriers has been estimated to be around 50 years, while *MLH1* and *MSH2* carriers are diagnosed on average 10 years earlier [34]. Moreover, mutations in the *MSH6* gene have also been linked to a lower risk of colorectal cancer and a higher risk of endometrial carcinoma [34]. Although germline mutations of *MSH6* gene in early onset colorectal cancer have been reported [35], this difference in age of onset and associated risk may explain why *MSH6* mutations constitute a minor fraction of cases.

In summary, as suggested by Jasperson et al. [11], the study of family cancer history, MSI, and IHC analyses followed by germline genetic testing represent an effective procedure for the diagnosis of Lynch syndrome in early onset cases.

Some population based studies showed that ~30% of biallelic *MUTYH* mutation carriers develop a colorectal cancer in the absence of a polyposis phenotype [13, 16, 35]. Accordingly, it has been suggested that *MUTYH* testing should be considered in early onset colorectal cancer patients with intact DNA MMR, regardless of family history or number of colonic polyps [13]. Giráldez et al. detected 2.8% of biallelic *MUTYH* mutations in a cohort of 140 patients with colorectal cancer diagnosed before the age of 50 [36]. In our study we performed systematic whole-gene sequencing

and did not find biallelic or monoallelic *MUTYH* mutations. These negative results could be explained by the limited number of investigated cases.

We identified only one pathogenetic mutation of *APC* in a 34-year-old patient with colorectal cancer and polyposis. The literature describes that Familial Adenomatous Polyposis is responsible for less than 1% of all colorectal cancers [37] and that the mean age for colorectal cancer development in this group of individuals is approximately 39 years [38], suggesting that also *APC* gene is implicated in cancer occurring at an early age.

In agreement with that reported in literature, we observed that 82% of early onset CRCs were not associated with known hereditary CRC syndromes [36]. This subset of tumors was mainly characterized by distal location, advanced stage, and predominance of the male gender as well as other investigators supported [39]. A previous study according to Ahnen et al. reported that cancer-specific survival in patients with young onset CRC is comparable to that of patients with late onset cancer [5, 38]. Moreover, we observed a similar frequency of somatic mutations for *KRAS* oncogene to overall sporadic colorectal cancers that correspond to 40% [40]. Regarding *BRAF* gene, a recent study suggests that *BRAF* mutations occur in 10–20% of sporadic colorectal cancer and are closely associated with the *MLH1* methylation. In our young patients, no *BRAF* mutations or *MLH1* promoter methylation was detected.

A second important aspect of our work concerns the analysis of aberrant hypo- and hyper-DNA methylation profiles of early onset CRCs compared with late onset CRCs. Both of these alterations are widely accepted as potential source of early biomarkers for diagnosis and prognosis in CRCs. CIMP phenotype, which accounts for almost 30–40% [41], has rarely been evaluated in early onset CRCs. In our work, no case with extensive gene methylation was observed among early CRCs and the average number of methylated genes was significantly lower in these tumors (both hereditary and nonhereditary CRCs) compared with the control group.

These results are in agreement with previous works that reported low levels of gene hypermethylation in Lynch syndrome cases [42, 43] as well as in nonhereditary early onset CRCs [19].

In our study, extensive gene hypermethylation was observed only in the late onset CRCs, accounting for 17% of these cases. In agreement with literature, this subset of CRCs frequently showed MSI and *BRAF* mutation and *MLH1* methylation [41, 43].

For the first time, we demonstrated that hypermethylation of three genes, namely, *ESR1*, *GATA5*, and *WT1*, was very common both in early onset (hereditary and nonhereditary tumors) and in late onset CRCs. Although these results need to be validated with further studies, our data have important clinical implications suggesting the usefulness of aberrant gene methylation analysis for the early detection and risk assessment of CRC, without using age at onset as a differential criterion. Promoter methylation analysis of serum and stool DNA has the potential to be used as a noninvasive test for the early diagnosis of CRC [44]. However, accurate selection of methylation markers is crucial for sensitive and specific detection of CRC as *de novo* methylation is also associated with aging [45]. *ESR1* is a well-known "type A" (age related) gene because its hypermethylation is demonstrated in both normal colorectal mucosa and CRCs, proportional to tissue age. By contrast, *GATA5* methylation has been reported as a suitable marker for early diagnosis of CRC [46] and its methylation is observed in colorectal adenomas but not in inflammatory colorectal tissues.

Moreover, our analysis has highlighted the potential utility of the *WT1* gene as an early diagnostic marker of CRC. To date, only a few studies have investigated *WT1* methylation, confirming our data of widespread methylation of this gene in CRCs [47, 48]. In our opinion, this finding deserves to be explored further, especially with respect to the aberrant mechanisms of loss of imprinting of 11p15 described in CRCs and the possible link between *WT1* methylation and the upregulation of *IGF2* transcription [49].

Genome-wide hypomethylation is also reported as an early event in CRC and it has been associated with the activation of protooncogenes (i.e., *MET*) [50] and the presence of chromosomal instability [51]. Recently, Antelo et al. [39] found significantly lower levels of LINE-1 methylation in early onset CRCs compared to late onset CRCs, suggesting that a high degree of LINE-1 hypomethylation is a unique feature of CRCs in young patients. At variance, our study does not confirm these previous findings, demonstrating very similar LINE-1 methylation levels in early onset and in late onset CRCs, with a normal distribution of LINE-1 values in both subsets of tumors. In our opinion, several factors may explain this discordance. Firstly, since the degree of LINE-1 demethylation prognosis is linear in relation to TNM-stage progression and this marker is a strong independent factor for poor prognosis [52], the evaluation of tumor stage is crucial when comparing different subsets of CRCs. The second important point regards the tumor location because lower levels of LINE-1 methylation have been reported in distal compared with proximal CRCs [39]. Antelo et al. examined two independent cohorts of CRCs developed ≤50 years including mainly advanced and distal CRCs without a matched selection of the late onset CRCs. For these reasons we designed a case-control study in which every early onset CRC was matched with a late onset CRC for sex, location, and stage. Finally, technical reasons may not be excluded, although the same methodological approach has been used in the two studies and very similar LINE-1 methylation levels were observed considering normal colorectal mucosa and MSI CRCs (both hereditary and sporadic tumors).

Moreover, Antelo et al. reported a similar result about higher levels of LINE-1 methylation in Lynch syndrome tumors than in group of early onset CRC. For this reason, it is maybe not optimal to use this testing for detection of early onset CRC, whereas Lynch syndrome is the most common hereditary CRC in young patients. Although our series of early onset CRCs is small and the present results are not conclusive about this issue, we believe that the strong positive association between LINE-1 hypomethylation and early onset CRC previously reported needs to be reconsidered through future larger case-control studies.

5. Conclusion

The results of this study can be summarised as follows.

First, Lynch syndrome is the most frequent cause of hereditary colorectal cancer in young patients; family cancer history, MSI, and IHC analyses followed by germline genetic testing represent the most appropriate procedure for Lynch syndrome diagnosis in early onset colorectal cancer. Second, early onset colorectal cancers with MMR deficiency were clinically and pathologically indistinguishable from colorectal MSS carcinomas. Third, epigenetic events (hyper- and hypomethylation) are not closely associated with early onset colorectal cancer. Finally, our study emphasises that the genetic basis in the majority of early onset colorectal carcinomas remains unknown. Further studies of the whole exome of this genetically undefined group of early onset colorectal tumors will need to elucidate possible pathogenetic mechanisms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work has been carried out with the financial support of Associazione Italiana Ricerca Cancro (AIRC) and the Associazione Ricerca Tumori Intestinali (ARTI). The authors gratefully acknowledge Maurizio Ponz de Leon for his expertise in hereditary colorectal cancer and Carmela Di Gregorio for her help in collecting samples and immunohistochemical analysis.

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