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EDITORIAL

Is transient elastography a useful tool for screening liver disease?

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Abstract

Transient elastography (TE) is a new non invasive tool for measuring liver stiffness, which is correlated to the histologic stage of liver fibrosis. Several studies in chronic liver disease (CLD) have determined a good accuracy of TE in predicting significant fibrosis and an optimal accuracy in predicting cirrhosis. Normal liver stiffness ranges between 3.3-7.8 KPa and using a cut off of 7.1 KPa, significant fibrosis and cirrhosis can be excluded with a very high negative predictive value (NPV). Positive predictive value (PPV) for the diagnosis of cirrhosis is lower using just a single scan but increases to 90% if high stiffness values are confirmed by a second independent scan. However the presence of fatty liver and metabolic syndrome slightly increases the readings and may reduce the accuracy of the test. It is uncertain if this increase is related to the presence of steatofibrosis or if it is caused by steatosis itself. TE can be used in screening patients attending the liver clinics to identify those with significant fibrosis or cirrhosis and may be particularly useful in discriminating HBV inactive carriers from chronic hepatitis B patients. TE, however, is not reliable in predicting the presence of esophageal varices in cirrhotics. Another potential indication for TE is the systematic screening of populations at high risk for CLD, such as intravenous drug users and alcoholics, but further studies are needed to determine its diagnostic accuracy in these settings.

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Key words: Transient elastography; Screening; Liver disease; Hepatitis B; Hepatitis C; Non alcoholic steatohepatitis; Cirrhosis

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INTRODUCTION

Transient elastography (TE) is a new non invasive tool for measuring liver stiffness, which is correlated to the histologic stage of liver fibrosis[1]. The device (Fibroscan) generates an elastic wave by means of a vibrator applied to the thoracic wall at the level of the right liver lobe. The vibrator produces a shot and a low amplitude shear wave propagating through the liver parenchyma. The velocity of propagation is directly proportional to liver stiffness and is automatically calculated by the instrument. The range of measurements, expressed in kilopascals, varies from 2.5 to 74 KPa.

Many studies have been published on the use of TE in patients with already diagnosed chronic liver disease (CLD) but few have addressed the issue of its possible use as a first line examination in the liver clinic or in facilities where patients at risk of liver disease are attending.

HOW TO TAKE AND INTERPRET THE MEASUREMENTS

In order to obtain valid and reproducible measurements the probe should be placed at the center of the right liver lobe, two intercostal spaces below the upper liver margin and at the level of the anterior or middle axillary line. If measurements are taken below this point and too close to the lower liver edge both the percentage of valid shots and the median stiffness tend to decrease[2]. Ultrasound assistance to locate the upper liver margin is usually unnecessary if the patient is reasonably thin because the liver can be recognized by percussion alone. The device incorporates an M-mode window enabling the operator to locate the liver parenchyma and avoid both ribs and lung. If the shot does not generate a readable wave the software classifies the measurement as unsuccessful. Liver stiffness

is defined as the median of 10 successful measurements and according to the manufacturer's recommendations at least 60% of the shots should be successful for each exam. The main reason for unsuccessful examination in the Western world is patients being overweight, while in the East intercostal spaces which are too narrow often hamper the appropriate contact of the probe. Overall failure rates in different studies range between 2.4% and 9.4%[3-6]. The presence of diabetes and being a transplant recipient have also been identified as independent predictors of failure in a recent study of 215 patients with CLD[7]. TE cannot be performed in ascitic patients because the interposed fluid blocks the progression of the shear wave. Other contraindications are pregnancy and the presence of a cardiac pacemaker because there are no safety studies on the use of TE in these conditions. TE is easy to perform, quick and reproducible although fatty liver and a low fibrosis stage may decrease reproducibility[3]. TE can also be easily learnt and performed by nurses[8] and the results are immediately available, thus saving physician's time and rendering this method particularly suitable for screening a large number of patients.

The validity of the results depends on one important parameter: the variability of measurements. This is reflected by the interquartile range (IQR), representing the range of values including 50% of patients above and below the median. According to the manufacturer's suggestion the IQR/median stiffness ratio should not exceed 30% of the median value, although it seems that 20% could assure the best concordance between liver biopsy and TE[9]. There are no studies specifically dealing with the problem of excessive variability of readings and therefore the interpretation of results is derived more from personal experience and from the manufacturer's advice than from observational data. It is still unknown if variability is observed only in diseased or also in normal livers and how this variability affects the interpretation of the test. The cause may be an improper examination technique or it may be inherent to the liver disease itself e.g. in macronodular cirrhosis stiffness may change in different areas of the liver. When variable readings are obtained it is important to check if the probe is perpendicular to the thoracic wall, that the vibrator is not touching against a rib and if the elastografic wave is straight and narrow. If the wave that has been generated is broad, bifid or angulated the software may reconstruct the velocity curve in different parts of the wave and give variable readings. It is important in these cases to obtain a "good" elastogram. This can be obtained by placing the probe in the middle of the right lobe and avoiding contact with the rib as that may dampen the shot and distort the shear wave.

WHAT ARE THE NORMAL VALUES OF TRANSIENT ELASTOGRAPHY?

Paradoxically many studies have been published in CLD patients, but only three in apparently normal subjects (Table 1): the first as a full paper[10] the second as a letter[11] and the third as an abstract[12]. In the first study, performed by Roulot, 429 apparently healthy subjects attending a free health check were studied by a single operator. Only values with an IQR/median stiffness of less than 30% were considered in the analysis, thus overcoming the problem of variability. Results could be obtained in 93.4% of the subjects, indicating that TE has a low failure rate in the general population. However, the percentage of failures rose to 25% in obese individuals (BMI > 30 kg/m^2) and 88% in morbid obese individuals, confirming that TE is not a good method for screening overweight people. This is a significant drawback, because many obese subjects have fatty livers and need a rapid, non invasive method to rule out significant fibrosis. Using the 5th and 95th centiles normal values were set between 3.3-7.8 KPa in women and 3.8-8 KPa in men. In the main studies of TE in chronic liver disease[13-18] the mean cut-off for significant fibrosis was established between 7 and 8 KPa, (Table 2), which is higher than the 95th centile of normal subjects. TE can thus reliably distinguish normal individuals from patients with significant fibrosis, although overlap exists with mild fibrosis. In addition none of the normal subjects studied by Roulot had values higher than 13-17 KPa, which is considered the cut off range for cirrhosis of all etiologies (Table 3).

Table 1 Liver stiffness in the normal population and factors influencing its measurement

	Corpechot C[11]	Roulot D[10]	Colombo S[12]
Number of subjects	71	429	327
Population	Healthy volunteers	Medical check-up	Blood donors
Mean stiffness (KPa)	4.8 (2.5-6.9)[1]	5.4 ± 1.5[2]	4.9 ± 1.7[2]
95th centile	-	8.6	7.8
Age	No effect	No effect	No effect
Gender	M > F	M > F	M = F
High BMI	Increased	Increased	Increased
Metabolic syndrome	-	Increased[3]	-
Fatty liver	-	-	Increased[3]

[1]Range; [2]Standard deviation; [3]At multivariate analysis.

Our group reproduced the same results in voluntary blood donors[12]: in the absence of fatty liver we observed a mean normal liver stiffness of 4.6 KPa ± 1.52 SD. Using 6.9 as the optimal cut off for normal individuals and comparing it with the cut offs from the literature, we obtained a 96% NPV for ruling out significant fibrosis and a 100% NPV for ruling out cirrhosis. In conclusion, normal subjects can be reliably differentiated from CLD patients. TE could thus be proposed as a good screening tool to detect significant fibrosis and as an optimal tool for the detection of cirrhosis, irrespective of the etiology.

DO STEATOSIS AND TRANSAMINASE LEVELS AFFECT THE READINGS?

An important finding of Roulot's paper is that mean stiffness was found to be 1.3 KPa higher in subjects with metabolic syndrome than in those without.

Table 2 Diagnostic performance of TE in the diagnosis of significant fibrosis

	Oliveri[14]	Marcellin[13]	Castera[35]	Ziol[15]	Fraquelli[3]	Corpechot[18]	Kelleher[16]	Yoneda[17]
Patients	268	170	183	251	200	95	129	67
F2 or higher (%)	69	50	74	65	50	60	50	49
Etiology	HBV	HBV	HCV	HCV	80% HCV	PBC/PSC	Nafld	Nafld
Cut Off (KPa)	7.5	7.2	7.1	8.8	7.9	7.3	8.7	6.6
Sensitivity (%)	93	70	67	56	72	84	81	82
Specificity (%)	88	83	89	91	84	87	78	81
AUROC	0.96	0.81	0.83	0.79	0.86	0.92	0.86	0.87

Table 3 Diagnostic performance of TE in the diagnosis of cirrhosis

	Olivieri[14]	Marcellin[13]	Castera[35]	Ziol[15]	Fraquelli[3]	Ganne-Carrié[5]	Corpechot[11]	Foucher[33]	Nguyen[36]	Yoneda[17]
Patients	268	202	183	251	200	775	95	354	103	67
Cirrhotics (%)	24	8	25	19	26	15	16	13	32	7.50
Etiology	HBV	HBV	HCV	HCV	80% HCV	All	PBC/PSC	All	Alcohol	Nafld
Cut-off (KPa)	11.8	11	12.5	14.6	11.9	14.6	17.3	17.6	19.5	17
Sensitivity (%)	86	93	87	86	91	79	93	77	91	93
Specificity (%)	96	87	91	96	89	95	95	97	100	95
AUROC	0.97	0.93	0.95	0.97	0.91	0.95	0.96	0.96	0.92	0.99

Metabolic syndrome was also the main predictor of increased stiffness after adjustment for age, sex, BMI and liver enzymes. This finding suggests that the normal ranges for liver stiffness should be shifted upwards in overweight patients with metabolic syndrome. However no ultrasound examination was performed in this study and therefore it was unknown if the increased stiffness was dependent on metabolic syndrome itself or on fatty liver. To answer this question we investigated 327 healthy blood donors using TE and abdominal ultrasound performed on the same day by two operators with good concordant readings[11]. Similarly to Roulot's study we had a very low failure rate (2.4%) confirming the good applicability of TE in population studies. At multiple regression analysis we found that the degree of steatosis, and not BMI, sex, age and liver enzymes, was related to liver stiffness. The central issue is whether steatosis itself increases liver stiffness or if it is caused by an underlying steatofibrosis. Data from the literature are inconclusive: in one study patients with chronic hepatitis C and the same fibrosis stage had increased liver stiffness if they had concomitant fatty liver[19]. In addition, there was a close relationship between the augmentation of liver stiffness and the degree of steatosis. However studies in chronic hepatitis B have shown that patients with the same stage of fibrosis had lower[20] or equal stiffness[21] in the case of accompanying steatosis. It seems unlikely that steatosis might influence liver stiffness in discordant ways depending on the type of hepatitis and therefore further studies are needed to clarify this issue.

Another possible confounding factor is the effect of transaminase (ALT) level. It is well known that acute hepatitis may spuriously cause extreme and transient elevations of liver stiffness[6,22,23], but also minor ALT elevations can alter TE readings and cause discordance with histological stage[24]. If elevated ALT may overestimate fibrosis stage the opposite is also true: e.g. elderly patients with normal or minimally elevated ALT may have their fibrosis stage underestimated. An algorithm has been proposed to correct for the underestimation of fibrosis in the elderly, but this algorithm has not yet been validated[24]. In conclusion, abdominal ultrasound and ALT determination should always be used together with TE in population screening.

NEW TECHNIQUES: REAL TIME ELASTOGRAPHY

From the above considerations it would be attractive to use a new device incorporating liver stiffness measurements with conventional ultrasound. This task could be accomplished by real time ultrasonography. This technique is performed with conventional ultrasound probes and equipment such as Hitachi EUB-8500 and EUB-900 machines. The examined tissue is divided into up to 30000 finite elements and compression is applied with the probe itself to the skin overlying the liver. During compression the displacement of each element is measured and recorded: in hard tissue the amount of displacement is low, whereas in soft tissue the amount of displacement is high. The calculation of soft tissue elasticity distribution is performed in real time and the results are presented in a colour-coded scale with a conventional B-mode image in the background. Liver stiffness can thus be determined during a conventional routine upper abdominal utrasonography. This new technique is rapid and cheaper than Fibroscan, but its accuracy should be tested against classic TE and liver biopsy. In one study comparing liver biopsy and real time ultrasonography the areas under the receiver operating curve (AUROC) were inferior to TE: 0.75 for equal or higher than F2 fibrosis, 0.73 for equal or higher than F3, 0.69 for F4 and APRI itself performed better than the new technique[25]. Using heart beats instead of manual

compression for displacement[26] may improve the accuracy and lead to better standardization. Clearly more studies are needed and at this time only TE has sufficient body of evidence to be proposed for screening studies.

WHEN TRANSIENT ELASTOGRAPHY CAN BE USED AS A FIRST LINE EXAMINATION

TE could theoretically be used to screen patients attending the liver clinic in order to identify: (a) patients with chronic hepatitis B and C with significant fibrosis, to establish an indication for antiviral therapy; (b) patients with non alcoholic fatty liver disease (NAFLD) or non alcoholic steatohepatitis with significant fibrosis in which aggressive dietary intervention or new therapies could be proposed; (c) patients with liver cirrhosis, in order to start sonographic surveillance for hepatocellular carcinoma; (d) patients with liver cirrhosis and significant portal hypertension, in order to start endoscopic surveillance of esophageal varices.

Outside the liver clinic TE could also be used to systematically screen populations at high risk for liver disease e.g. intravenous drug addicts or alcoholic patients attending rehabilitation programs.

In the clinical setting accuracy for the diagnosis of cirrhosis is higher than for significant fibrosis with a median AUROC of 0.95 *vs* 0.86 irrespective of the etiology of liver disease (Tables 2 and 3). The best accuracy is achieved in ruling out cirrhosis, with a NPV close to 100%[1]. It should be stressed that despite similar AUROCs, cut offs for significant fibrosis and cirrhosis vary according to etiologies, being lower for hepatitis B, intermediate for hepatitis C and higher for NAFLD or alcoholic liver disease. In chronic hepatitis C and NAFLD there is a continuous spectrum of fibrosis irrespective of ALT levels and therefore it would be preferable to use ranges of values instead of cut-offs[1]. On the contrary, in hepatitis B virus infection there is no spectrum of continuity between the inactive carrier state and the chronic hepatitis B patient[27] and the use of a cut off value would be appropriate. TE can reliably differentiate the inactive carrier from chronic hepatitis B. In a recent paper[14] the mean stiffness value of the liver of an inactive carrier was found to be 5 KPa ± 1.8 SD, which is similar to normal controls and different from chronic hepatitis B patients with significant fibrosis. Therefore, if a patient suspected to be an inactive carrier has normal stiffness and elevated ALT, another cause for the increased ALT levels should be sought e.g. concomitant NAFLD. The capacity of correctly identifying chronic HBV carriers could be of immense value in regions with high prevalence of HBsAg, where it could be used together with ALT measurement as a quick and cheap screening test for a large proportion of the population.

If TE is a useful tool to diagnose significant fibrosis and cirrhosis in CLD patients and to define the inactive HBsAg carrier, it is not so for predicting portal hypertension and esophageal varices. In fact a good correlation between stiffness and hepatic-vein portal gradient (HVPG) was found only up to HVPG values of 10-12 mmHg, whereas for higher values the correlation was suboptimal[28]. This could be explained by the fact that TE measures the initial rise of portal pressure caused by the accumulation of a fibrillar matrix, but not the complex hemodynamic changes of late portal hypertension[29]. Accordingly TE was not accurate in prediction of esophageal varices, with an AUROC ranging from 0.76 to 0.84 in various studies[29-31]. Although sensitivity was good (71%-96%) , specificity and positive predictive values (PPV) were low (60%-80% and 48%-54%) and overall accuracy was inferior compared to simple tests like platelet count/spleen diameter ratio[32]. Another problem arising from these studies is the wide range of proposed cut offs, varying from 13.9 to 21.3 KPa for all varices and from 19 to 30 KPa for F2 varices[30,31]. The optimal cut offs therefore are still to be defined.

It would also be interesting to determine a cut off for liver stiffness associated with an increased risk for hepatocellular carcinoma, thus warranting enhanced surveillance for this type of patient. This issue was addressed in only one study[33] in which 144 patients with cirrhosis or advanced fibrosis of varying etiologies were studied with TE and appropriate imaging. According to the authors, a cut of 53.7 KPa could identify cirrhotics harbouring hepatocellular carcinoma with good specificity (87%) and high NPV (90%). PPV and sensitivity were however too low (around 30%) to propose TE as a screening tool for determining the risk of hepatocellular carcinoma. Moreover the conclusions were drawn from only 19 liver cancer patients and clearly more studies are needed in larger cohorts of patients.

In different settings from liver clinics, TE has been studied in IVDU[34] and alcoholics participating in a rehabilitation program (Melin P, personal communication). In the first study, conducted in Denmark, 434 IVDU from 6 methadone clinics were studied[34]. Among the 394 subjects in which TE could be performed, 11 % had cirrhosis (> 12 KPa) and 16% significant fibrosis (8-12 KPa). Twenty-five patients with stiffness > 12 KPa had a repeated TE measurement at the time of liver biopsy. It is interesting to note that 6 patients had a stiffness < 12 KPa at the second scan and all of them had mild fibrosis at biopsy, while only 1 out of 19 patients confirmed at the second scan had mild fibrosis. The authors conclude that two consecutive and concordant scans are needed in order to establish a confident diagnosis of cirrhosis. In fact, PPV for the diagnosis of cirrhosis increased from 50% to 94% after 2 independent scans. The take home message of this study is that in population screening, it is advisable to confirm all elevated results with a second independent scan.

CONCLUSION

In conclusion, there are not enough data to recommend TE as a screening tool outside of liver clinics and

specific studies are needed on high risk populations. On the basis of existing evidence we can conclude that TE has a high NPV to exclude cirrhosis. PPV is low with a single scan but can increase to 90% if high stiffness values are confirmed by a second independent scan. Accuracy for diagnosing significant fibrosis is lower than for cirrhosis and different cut offs must be taken into account. However, TE is not useful for the prediction of esophageal varices, because PPV is low and cut-offs are still undefined.

REFERENCES

1 **Castera L**, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol* 2008; **48**: 835-847

2 **Abergel A**, Bonny C, Randl K, Nicolas C, Roszyk L, Noirfalise C, Massoulier S, Chauterame B, Sapin V, Bommelaer G. Fibroscan measures according to intercostal space: validity andconcordance. *J Hepatol* 2008; **48** Suppl 2: S268

3 **Fraquelli M**, Rigamonti C, Casazza G, Conte D, Donato MF, Ronchi G, Colombo M. Reproducibility of transient elastography in the evaluation of liver fibrosis in patients with chronic liver disease. *Gut* 2007; **56**: 968-973

4 **Castera L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

5 **Ganne-Carrie N**, Ziol M, de Ledinghen V, Douvin C, Marcellin P, Castera L, Dhumeaux D, Trinchet JC, Beaugrand M. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. *Hepatology* 2006; **44**: 1511-1517

6 **Coco B**, Oliveri F, Maina AM, Ciccorossi P, Sacco R, Colombatto P, Bonino F, Brunetto MR. Transient elastography: a new surrogate marker of liver fibrosis influenced by major changes of transaminases. *J Viral Hepat* 2007; **14**: 360-369

7 **Marin-Gabriel JC**, De-la-Cruz JB, Tocado M, Rodriguez-Gil Y, Fernandez-Vasquez I, Manzano-Alonso ML, Martin-Algibez AM, Meneu-Diaz JC, Coline-Ruiz Delgado F, Moreno-Gonzales E, Solis-Herruzo JA, Castellano-Tertajada G. Failure of liver stiffness measurement with fibroscan: prevalence and determinants. *J Hepatol* 2008; **48** Suppl 2: S279

8 **Kettaneh A**, Marcellin P, Douvin C, Poupon R, Ziol M, Beaugrand M, de Ledinghen V. Features associated with success rate and performance of FibroScan measurements for the diagnosis of cirrhosis in HCV patients: a prospective study of 935 patients. *J Hepatol* 2007; **46**: 628-634

9 **Lucidarme D**, Foucher J, Le Bail B, Castera L, Villars S, Forzy G, Filoche B, Couzigou P, deLedinghen V. Ratio interquartile range / median value of liver stiffness measurement is a key factor of accuracy of transient elastography (FIBROSCAN®) for the diagnosis of liver fibrosis. *Hepatology* 2007; **46** Suppl: A318

10 **Roulot D**, Czernichow S, Le Clesiau H, Costes JL, Vergnaud AC, Beaugrand M. Liver stiffness values in apparently healthy subjects: influence of gender and metabolic syndrome. *J Hepatol* 2008; **48**: 606-613

11 **Corpechot C**, El Naggar A, Poupon R. Gender and liver: is the liver stiffness weaker in weaker sex? *Hepatology* 2006; **44**: 513-514

12 **Colombo S**, Belloli L, Buonocore M, Jamoletti C, Zaccanelli M, Badia E, Del Poggio P. Liver Stiffness values in the normal population: a studying voluntary blood donors. *Hepatology* 2008; **48** Suppl: A995

13 **Marcellin P**, de Ledinghen V, Dhumeaux D, Poupon R, Ziol M, Bedossa P, Beaugrand M. Non invasive assessment of liver fibrosis in chronic hepatitis B using Fibroscan. *Hepatology* 2005; **42** Suppl: A715

14 **Oliveri F**, Coco B, Ciccorossi P, Colombatto P, Romagnoli V, Cherubini B, Bonino F, Brunetto MR. Liver stiffness in the hepatitis B virus carrier: A non-invasive marker of liver disease influenced by the pattern of transaminases. *World J Gastroenterol* 2008; **14**: 6154-6162

15 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54

16 **Kelleher T**, MacFarlane C, de Ledinghen V, Beaugrand M, Foucher J, Castera L. Risk factors and hepatic elastography (FibroScan) in the prediction of hepatic fibrosis in non-alcoholic steatohepatitis. *Gastroenterology* 2006; **130**: A736

17 **Yoneda M**, Yoneda M, Fujita K, Inamori M, Tamano M, Hiriishi H, Nakajima A. Transient elastography in patients with non-alcoholic fatty liver disease (NAFLD). *Gut* 2007; **56**: 1330-1331

18 **Corpechot C**, El Naggar A, Poujol-Robert A, Ziol M, Wendum D, Chazouilleres O, de Ledinghen V, Dhumeaux D, Marcellin P, Beaugrand M, Poupon R. Assessment of biliary fibrosis by transient elastography in patients with PBC and PSC. *Hepatology* 2006; **43**: 1118-1124

19 **Lupsor M**, Stefanescu H, Sparchez Z, Serban A, Grigorescu M, Iancu S, Suten T, Badea R. The influence of fatty load on liver stiffness in chronic hepatitis C patients. *J Hepatol* 2008; **48** Suppl 2: S278

20 **Gaia S**, Carenzi S, Brunello F, Barilli AL, Lagger M, Bugianesi E, Smedile A, Rizzetto M. Is liver stiffness measurement different in patients with NASH or with viral hepatitis? *Dig Liver Dis* 2008; **40**: A128

21 **Kim SU**, Kim DY, Park JY, Ahn SH, Paik YH, Choi EH. The impact of steatosis on liver stiffness measurement using fibroscan in patients with hepatitis B virus related chronic liver disease in Korea. *Hepatology* 2008; **48**: Suppl 4: A994

22 **Arena U**, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S, Moscarella S, Boddi V, Petrarca A, Laffi G, Marra F, Pinzani M. Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380-384

23 **Sagir A**, Erhardt A, Schmitt M, Haussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. *Hepatology* 2008; **47**: 592-595

24 **Calvaruso V**, Cammà C, Di Marco V, Maimone S, Bronte F, Enea M, Pleguezuelo M, Xirouchakis E, Misseri M, Manousou P, Dusheiko M, Burroughs A, Craxi A. Error factors for transient elastography in chronic hepatitis C. *Hepatology* 2008; **48** Suppl: A313

25 **Friedrich-Rust M**, Ong MF, Herrmann E, Dries V, Samaras P, Zeuzem S, Sarrazin C. Real-time elastography for noninvasive assessment of liver fibrosis in chronic viral hepatitis. *AJR Am J Roentgenol* 2007; **188**: 758-764

26 **Yoshioka K**, Kawabe N, Hashimoto S. Transient elastography: Applications and limitations. *Hepatol Res* 2008; **38**: 1063-1068

27 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539

28 **Vizzutti F**, Arena U, Romanelli RG, Rega L, Foschi M, Colagrande S, Petrarca A, Moscarella S, Belli G, Zignego AL, Marra F, Laffi G, Pinzani M. Liver stiffness measurement predicts severe portal hypertension in patients with HCV-related cirrhosis. *Hepatology* 2007; **45**: 1290-1297

29 **Lim JK**, Groszmann RJ. Transient elastography for diagnosis of portal hypertension in liver cirrhosis: is there still a role for hepatic venous pressure gradient measurement? *Hepatology* 2007; **45**: 1087-1090

30 **Castera L**, Bernard PH, Le Bail B, Foucher J, Merrouche W,

Couzigou P, de Ledinghen V. What is the best non invasive method for early prediction of cirrhosis inchronic hepatitis C? Prospective comparison between Fibroscan and serum markers. *Hepatology* 2007; **46** Suppl: A581

31 **Kazemi F**, Kettaneh A, N'kontchou G, Pinto E, Ganne-Carrie N, Trinchet JC, Beaugrand M. Liver stiffness measurement selects patients with cirrhosis at risk of bearing large oesophageal varices. *J Hepatol* 2006; **45**: 230-235

32 **Giannini E**, Botta F, Borro P, Risso D, Romagnoli P, Fasoli A, Mele MR, Testa E, Mansi C, Savarino V, Testa R. Platelet count/spleen diameter ratio: proposal and validation of a non-invasive parameter to predict the presence of oesophageal varices in patients with liver cirrhosis. *Gut* 2003; **52**: 1200-1205

33 **Foucher J**, Chanteloup E, Vergniol J, Castera L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Ledinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408

34 **Klemmensen Mossner B**, Riis Jorgensen T, Skamling M, Pedersen C, Christensen PB. Outreach screening of drug users with fibroscan identifies a high proportion of severe fibrosis not previously recognized. *J Hepatol* 2008; **48** Suppl 2: S276

35 **Castera L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

36 **Nguyen-Khac E**, Chatelain D, Tramier B, Decrombecque C, Robert B, Joly JP, Brevet M, Grignon P, Lion S, Le Page L, Dupas JL. Assessment of asymptomatic liver fibrosis in alcoholic patients using fibroscan: prospective comparison with seven non-invasive laboratory tests. *Aliment Pharmacol Ther* 2008; **28**: 1188-1198

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Isolated segmental, sectoral and right hepatic bile duct injuries

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Abstract

The treatment of isolated segmental, sectoral and right hepatic bile duct injuries is controversial. Nineteen patients were treated over a 26-year period. Group one was comprised of 4 patients in whom the injury was primarily repaired during the original surgery; 3 over a T-tube, 1 with a Roux-en-Y. These patients had an uneventful recovery. The second group consisted of 5 patients in whom the duct was ligated; 4 developed infection, 3 of which required drainage and biliary repair. Two patients had good long-term outcomes; the third developed a late anastomotic stricture requiring further surgery. The fourth patient developed a small bile leak and pain which resolved spontaneously. The fifth patient developed complications from which he died. The third group was comprised of 4 patients referred with biliary peritonitis; all underwent drainage and lavage, and developed biliary fistulae, 3 of which resolved spontaneously, 1 required Roux-en-Y repair, with favorable outcomes. The fourth group consisted of 6 patients with biliary fistulae. Two patients, both with an 8-wk history of a fistula, underwent Roux-en-Y repair. Two others also underwent a Roux-en-Y repair, as their fistulae showed no signs of closure. The remaining 2 patients had spontaneous closure of their biliary fistulae. A primary repair is a reasonable alternative to ligature of injured duct. Patients with ligated ducts may develop complications. Infected ducts require further surgery. Patients with biliary peritonitis must be treated with drainage and lavage. There is a 50% chance that a biliary fistula will close spontaneously. In cases where the biliary fistula does not close within 6 to 8 wk, a Roux-en-Y anastomosis should be considered.

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Key words: Segmental bile duct; Sectoral bile duct; Right hepatic bile duct; Injury; Treatment

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INTRODUCTION

Dangerous anatomy, dangerous pathologies and dangerous surgery are all factors which lead to bile duct injuries in both open and laparoscopic cholecystectomy[1] Dangerous anatomical variants of the right liver bile ducts are known to occur in around 15%-20% of patients, and may be injured during cholecystectomy. These operative sectoral and segmental bile ducts injuries (SSBDI) can often pass by unnoticed and without serious symptoms, and thus the frequency is likely to be far higher. They can however also lead to serious complications such as cholangitis and liver abscesses or biliary fistula and peritonitis.

The treatment of SSBDI is controversial, and the different approaches often depend on the timing of the diagnosis and the types of complications. This study reviews our management of SSBDI.

ISOLATED SEGMENTAL, SECTORAL AND RIGHT HEPATIC BILE DUCT INJURIES

Dangerous anatomy, dangerous pathologies and dangerous surgery are all factors which lead to bile duct injuries in both open and laparoscopic cholecystectomy[1]. There are three main dangerous anatomical variants. Firstly, the cystic duct may be near to the segmental (Figure 1A), or sectoral bile duct (Figure 1B) so that these ducts may be injured during dissection, transection or occlusion of the cystic duct. Secondly, the

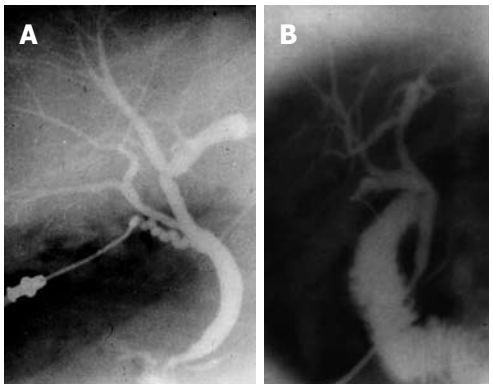

Figure 1 Cystic duct. A: near a segmental bile duct; B: very close to the sectoral bile duct.

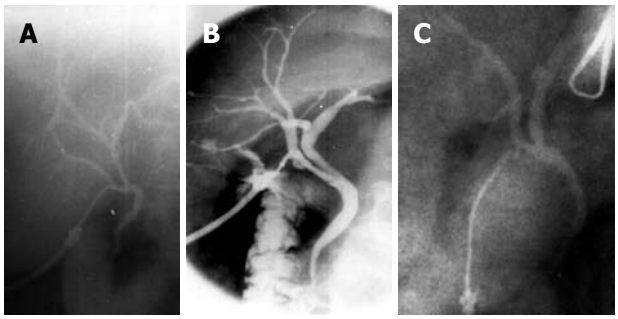

Figure 2 Cystic duct. A: Cystic duct joining the segmental; B: Sectoral hepatic ducts that could be misinterpreted as cystic ducts; C: Right hepatic ducts that could be misinterpreted as cystic ducts.

cystic duct may join one of these ducts, instead of the common bile duct (Figure 2A-C) so that one of them may be misinterpreted as a cystic duct and so transected, resected and occluded. Thirdly, the cystic duct may join the convergence of the sectoral or hepatic ducts (Figure 3A and B) so that the common bile duct may be misinterpreted as a cystic duct and so transected and resected.

Dangerous anatomical variants of the right liver bile ducts are known to occur in around 15%-20% of patients, and may be injured during cholecystectomy. These operative isolated segmental, sectoral and right hepatic bile duct injuries can pass by unnoticed and without serious symptoms, and thus the frequency is likely to be even greater. They can however also lead to serious complications such as cholangitis and liver abscesses or biliary fistula and peritonitis. The treatment of isolated segmental, sectoral and right hepatic bile duct injuries is controversial, and the different approaches often depend on the timing of the diagnosis and the types of complications.

We retrospectively reviewed the management of isolated segmental, sectoral and right hepatic bile duct injuries treated in our centre over a 26 year period (1982-2008). The 19 patients (14 women, with a mean age of 51 years) were divided into 4 groups in order to better analyze the different approaches (Table 1). The first group was comprised of 4 patients in whom the injury was recognized at the time of original surgery. Each patient

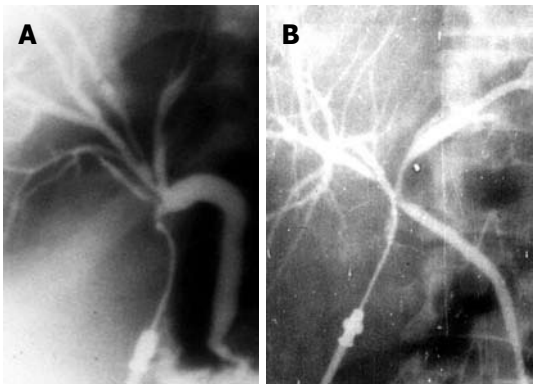

Figure 3 Cystic duct joins the convergence of the sectoral (A) and hepatic ducts (B).

underwent a primary repair, 3 over a tiny T-tube inserted into the common bile duct and then passed through the anastomosis, and 1 patient had an anastomosis of the injured duct with a Roux-en-Y jejunal limb (Figure 4). All the 4 patients had an uneventful recovery and continue to remain well to this day.

The second group consisted of 5 patients in whom the injured duct was ligated. Four of the patients developed cholangitis, 2 of them then also developed liver abscesses requiring drainage and biliary repair. Two patients had good long-term outcomes, the third developed a late anastomotic stricture which was then successfully resolved by further surgery, while the fourth patient developed a small temporary bile leak and pain which resolved spontaneously and required no further treatment. The fifth patient developed serious complications (a liver abscess, an abdominal wound disruption, and pneumonia) from which he finally died.

The third group was comprised of 4 patients referred from other hospitals with neglected biliary peritonitis which had lasted for several weeks. All the 4 patients underwent laparotomy, drainage and lavage. All the 4 developed an external biliary fistula through the subhepatic drain, 3 of which resolved spontaneously, and 1 of which required Roux-en-Y repair. All 4 patients had good long-term outcomes.

The final group consisted of 6 patients with external biliary fistulae which had lasted for between 2 and 8 wk, also referred from other hospitals. Two patients, both with an 8 wk history of a fistula, underwent immediate Roux-en-Y repair. Two other patients also underwent a Roux-en-Y repair, as their fistulae showed no signs of spontaneous closure (Figure 5). The remaining 2 patients had spontaneous closure of their biliary fistulae and required no further treatment (Figure 6).

Thus 12 (63%) patients in this series underwent some form of repair, 3 over T-tube and 9 with Roux-en-Y anastomosis. Repair was not necessary in 6 (32%) patients. 5 of the 10 patients with biliary fistula formation required Roux-en-Y repair, while 5 had spontaneous resolution. One patient (5%) who had duct ligation died.

No single method of treatment for isolated

Table 1 Patient demographics including gender, age, intraoperative recognition of the bile duct injuries, immediate repair and the method utilised, complications, any treatment of the complications, delayed treatments, long-term outcomes and late complications

Patient number	Gender	Age	Injury recognised intra-op	Immediate intervention	Complication	Treatment for complication	Delayed treatment	Outcome	Late complication
1	F	60	Yes	Repair over a T tube	No	No	None	Excellent	None
2	M	54	Yes	Repair over a T tube	No	No	None	Excellent	None
3	F	45	Yes	Roux-en-Y	No	No	None	Excellent	None
4	M	76	Yes	Repair over a T tube	Wound infection	No	None	Good	Hernia
5	M	62	Yes	Ligature	Liver abscess peritonitis	Drainage	None	Died	None
6	F	65	Yes	Ligature	Cholangitis	Roux-en-Y	None	Excellent	None
7	F	45	No	Ligature	Pain, fever, liver abscess	Roux-en-Y	Roux-en-Y	Good	None
8	F	48	No	Ligature	Pain, fever	Roux-en-Y	None	Excellent	None
9	F	60	No	Ligature	Temp pain	No	None	Good	None
10	F	28	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	None
11	F	24	No	None	Biliary peritonitis	Lavage, drainage	Roux-en-Y	Excellent	None
12	F	65	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	Hernia
13	F	30	No	None	Biliary peritonitis	Lavage, drainage	None	Closure	Ileus
14	F	21	No	None	Ext. fistula	No	None	Closure	None
15	F	36	No	None	Ext. fistula	No	None	Closure	None
16	F	60	No	None	Ext. fistula	No	Roux-en-Y	Excellent	None
17	M	60	No	None	Ext. fistula	No	Roux-en-Y	Good	None
18	F	63	No	None	Ext. fistula	Roux-en-Y	None	Excellent	None
19	M	73	No	None	Ext. fistula	Roux-en-Y	None	Excellent	None

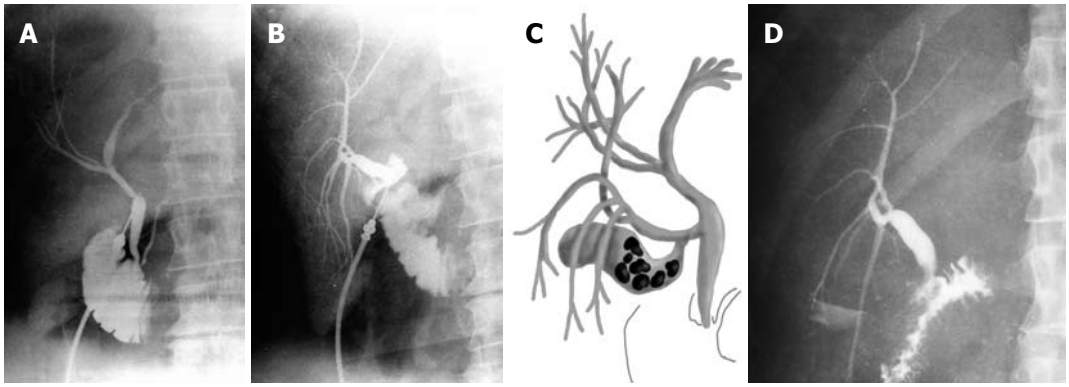

Figure 4 Cholangiography. A: An operative cholangiography with missing ducts within segments Ⅴ and Ⅷ; B: Cholangiography of the injured anterior sectoral duct; C: A schematic of the biliary anatomy before the injury took place (the anterior sectoral duct was misinterpreted as a cystic duct); D: Following hepatico-jejunostomy.

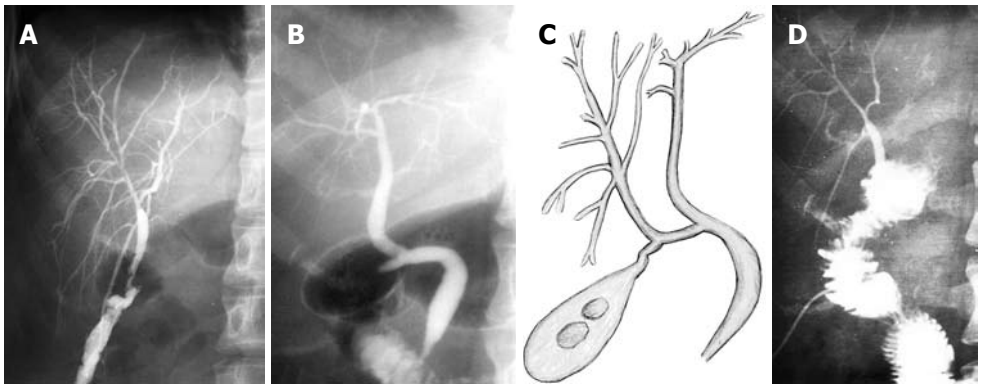

Figure 5 Demonstrates a case with an external biliary fistula that required Roux-en-Y repair. A: Fistulography showing the right hepatic duct; B: ERCP showing the rest of the biliary tree; C: A schematic of the biliary anatomy before the injury took place (the right hepatic duct was misinterpreted as a cystic duct); D: Following hepatico-jejunostomy.

segmental, sectoral and right hepatic bile duct injuries is universally accepted. Some authors believe that due to the uncertain outcomes and potentially harmful complications of reconstructive surgery, simple ligation of injured ducts is the treatment of choice. This is felt to be the case irrespective of the size of the injured duct[12], as unobstructed drainage of up to 50% of an otherwise normal liver, through either the right or left

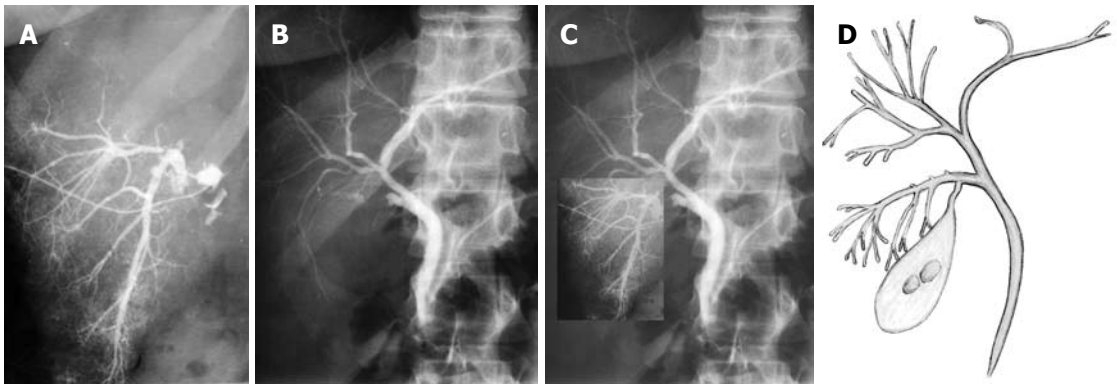

Figure 6 A biliary fistula that resolved spontaneously. A: Fistulography showing the ducts within segment V; B: ERCP showing the rest of the biliary tree; C: The entire biliary tree if the injury hadn't take place; D: A schematic of the biliary anatomy prior to injury.

unaffected ducts, is adequate to restore normal liver function; even with the obstructed lobe remaining in situ[3,4]. The non-draining lobe of the liver would then undergo progressive asymptomatic atrophy, and not require further treatment; so long as it was completely obstructed and free from infection, and that there was normal biliary flow from the residual 50% of the liver[3-5]. Although the incidence of "free communication" between the two main hepatic ductal systems above the hilum has been reported in up to 50% of patients[6], there is no evidence that the bile from an obstructed ductal system drains through the other unobstructed ducts. Other authors believe that wherever possible isolated segmental, sectoral and right hepatic bile duct injuries should be immediately repaired if recognized, in order to restore normal anatomy and function[7-9].

We favour a primary repair whenever an injury is recognized at original surgery. An end-to-end anastomosis over a tiny T-tube inserted in the common bile duct can be performed if the duct is of a reasonable size (at least 4 mm). There must be no loss of duct tissue, ductal tears or thermal injury, and the repair must be technically perfect using interrupted 5/0 slow-absorbable sutures. We believe that the end-to-end anastomosis is not indicated in injuries close to the common bile duct as any eventual stricture of the anastomosis may cause a stricture of the main bile duct. A Roux-en-Y hepaticojejunostomy can be performed in other situations and where the remaining duct is large enough to accommodate sutures.

Patients with isolated segmental, sectoral and right hepatic bile duct injury have also been successfully treated by nonsurgical methods such as endoscopic and/or percutaneous drainage and stenting[10]. In some rare cases with high hepatic injuries segmental liver resection has even been performed in order to avoid long-term transhepatic stenting and its complications such as cholangitis and late stricture formation[11].

Although surgeons have different approaches to the treatment of isolated segmental, sectoral and right hepatic bile duct injury recognized during or soon after the original surgery, there is no major disagreement in the treatment of cases with infection, biliary peritonitis and biliary fistula. Infection is treated stepwise, initially with antibiotics and percutaneous biliary drainage where possible, and if required with a Roux-en-Y repair[5] or even with hepatic resection of the infected parenchyma and ducts[3]. Biliary peritonitis requires drainage and lavage, previously by laparotomy and now more commonly by laparoscopy, in order to prevent abdominal abscesses[12]. Biliary fistulae are treated conservatively. If they fail to resolve spontaneously then a biliary repair with a Roux-en-Y may be necessary[13].

Thus in conclusion, a primary repair, where possible, is a reasonable solution for isolated segmental, sectoral and right hepatic bile duct injury recognized during the original surgery; the larger the duct the greater need for primary repair. A patient with a ligated duct may develop an infection, and so should be closely followed up for several weeks. Infected ducts require further surgery and usually a Roux-en-Y repair. In the past, patients with biliary peritonitis were treated with open laparotomy, drainage and lavage of the abdominal cavity. Nowadays, lavage and drainage can be successfully carried out laparoscopically or even percutaneously. Biliary fistulae should be followed for several weeks as there is a 50% chance of spontaneous closure. In cases where the biliary fistula does not close within 6 to 8 wk, the patient should probably undergo a Roux-en-Y anastomosis without further delay.

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REFERENCES

1 **Johnston GW**. Iatrogenic bile duct stricture: an avoidable surgical hazard? *Br J Surg* 1986; **73**: 245-247
2 **Hadjis NS**, Blumgart LH. Injury to segmental bile ducts. A reappraisal. *Arch Surg* 1988; **123**: 351-353
3 **Longmire WP Jr**, Tompkins RK. Lesions of the segmental and lobar hepatic ducts. *Ann Surg* 1975; **182**: 478-495
4 **Morrison CP**, Wemyss-Holden SA, & Maddern GJ, Successful management of iatrogenic bile duct injury by laparoscopic ligation. *HPB* 2003; **5**: 54-57
5 **Lillemoe KD**, Petrofski JA, Choti MA, Venbrux AC,

Cameron JL. Isolated right segmental hepatic duct injury: a diagnostic and therapeutic challenge. *J Gastrointest Surg* 2000; **4**: 168-177

6 **Baer HU**, Rhyner M, Stain SC, Glauser PW, Dennison AR, Maddern GJ, Blumgart LH. The effect of communication between the right and left liver on the outcome of surgical drainage for jaundice due to malignant obstruction at the hilus of the liver. *HPB Surg* 1994; **8**: 27-31

7 **Knight M**. Abnormalities of the gallbladder, bile ducts and arteries. In: Marlow S, Sherlock S, editors. Surgery of the gallbladder and bile ducts. 2nd ed. London: Butterworth, 1981: 97-116

8 **Braasch JW**. Segmental surgical disease of the liver. *Ann Surg* 1968; **168**: 110-115

9 **Thompson RW**, Schuler JG. Bile peritonitis from a cholecystohepatic bile ductule: an unusual complication of cholecystectomy. *Surgery* 1986; **99**: 511-513

10 **Perini RF**, Uflacker R, Cunningham JT, Selby JB, Adams D. Isolated right segmental hepatic duct injury following laparoscopic cholecystectomy. *Cardiovasc Intervent Radiol* 2005; **28**: 185-195

11 **Lichtenstein S**, Moorman DW, Malatesta JQ, Martin MF. The role of hepatic resection in the management of bile duct injuries following laparoscopic cholecystectomy. *Am Surg* 2000; **66**: 372-376; discussion 377

12 **Colovic R**, Barisic G, Markovic V. [Long-term results of treatment of injuries of the sectoral and segmental bile ducts] *Srp Arh Celok Lek* 2003; **131**: 314-318

13 **Czerniak A**, Thompson JN, Soreide O, Benjamin IS, Blumgart LH. The management of fistulas of the biliary tract after injury to the bile duct during cholecystectomy. *Surg Gynecol Obstet* 1988; **167**: 33-38

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REVIEW

Individually administered or co-prescribed thiopurines and mesalamines for inflammatory bowel disease

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Abstract

Data from both basic research and clinical experience continue to suggest that mesalamines and thiopurines are effective and efficient for the maintenance of remission of inflammatory bowel diseases. Several decades following the formalization of their indications, attention on these two drugs has been fostered by recent achievements. Demonstration of the ability of mesalamine to activate a colonocyte differentiation factor has shed light on its chemopreventive effects on colorectal cancer; in addition to their anti-proliferative efficacy, thiopurines have been shown to be specific regulators of apoptosis. The two drugs are often co-administered in clinical practice. Recent advancements have shown that mesalamines exert a positive synergism in this context, insofar as they can inhibit side-methylation of thiopurines and hasten the function of the main immunosuppressive pathways. Considering that up to 40% of patients cannot tolerate thiopurines, such renovated targets have stimulated efforts to improve compliance by research on the toxicity mechanisms. The definition of genetic polymorphisms in the enzymes of thiopurine metabolism, and the uncovering of synergistic drug interactions, such as that with allopurinol, are just two of the results of such efforts. Interaction between basic research and clinical practice has continued to inform indications and refine the prescriptions of mesalamines and thiopurines; these have not been restrained (they have been implemented in some cases) by the advent of the novel biological molecules with anti-cytokine activity.

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INTRODUCTION

Maintenance of remission of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) is a crucial target for at least two reasons: unchecked bowel inflammation behaves as an independent factor in colon cancer development[1]; and the inflammatory pathways do mutate in relapsing inflammatory bouts, thus favoring development of drug unresponsiveness and activation of apoptosis-resistant non-professional immunocytes[2]. Two classes of drugs form the traditional arsenal for IBD remission maintenance: mesalamines and thiopurines, and the remainder of this review emphasizes that they are far from being set aside. Mesalamines and thiopurines share a few facts in common: they have both been studied in the second half of the last century for indications that were eventually changed; they were studied by two female scientists, one of them was awarded the Nobel prize; they are both effective and cheap; and they both continue to remain under the limelight as novel pharmacological actions are being uncovered beyond those already known. Mesalamines and thiopurines are

often co-prescribed, and among other topics, we shall review the pros and cons of this association.

MESALAMINES

Brief history

The ancestor of mesalamine is a compound named salazopyrin (SASP) made by a sulfamidic moiety linked by an azo bond to 5-aminosalycilic acid (5-ASA). Such combined anti-bacterial and anti-inflammatory actions came to the attention of the Swedish rheumatologist Nanna Svartz, who believed that the joint lesions in her patients may have been caused by latent infections and inflammation. In the early 1940s, while giving SASP to her rheumatic patients, to take advantage of its double actions, she noticed an improvement in IBD in co-morbid subjects. Her observations were reported in 1942[3] and were to be confirmed in a controlled fashion 20 years later[4]. The abandonment of the infectious theory in rheumatoid arthritis and the acknowledgement that the significant allergic toxicity of SASP was mainly caused by its sulfa moiety led to the development of 5-ASAs compounds in the following decades, which are known as mesalazine in Europe and as mesalamine in the US.

Pharmacology, metabolism, and mechanism of action of 5-ASA

Upon ingestion, 5-ASA is partially oxidized in the stomach, absorbed in the proximal gut, and acetylated in the liver. Although it is not as efficiently absorbed in the terminal gut, the efficiency of the above described processes causes a significant portion of 5-ASA to be transferred to the bloodstream, thus posing the need for an efficient carrier to effect drug delivery to distal areas of disease. Diverse ways to address the need for the distal delivery of 5-ASA have been pursued in the last decades: use of a pH-dependent carrier that releases the active drug distal to the ileo-cecal valve, an ethyl-cellulose capsule to release 5-ASA evenly in the digestive lumen, or, the synthesis of dimers in which the azo bond is supposed to be broken by the colonic flora[5]. *In vitro*, 5-ASA has been shown to share several pharmacological properties with non-steroidal anti-inflammatory compounds, including: inhibition of nuclear factor (NF)-κB-dependent inflammatory pathways[6]; limitation of the oxidative stress in epithelial cells[7,8]; increase in the cellular heat-shock protein response[9]; inhibition of leukotriene production[10]; and modulation of prostaglandin metabolism[11].

Indications

The indications for mesalamines do differ between UC and CD. At least one study[12] has claimed that SASP is superior to placebo in the treatment of active left-sided CD. On the contrary, the results are mixed as to the indication for remission maintenance; a recent review[13] has recommended that prescription of mesalamines for the maintenance of CD be avoided. Regarding the management of UC, there are different data. In one large study[14], mesalamine doses ranging between 1 and 4 g/d have been shown to induce remission in 30% of patients, as compared with 12% remission achieved by placebo. A recent Cochrane[15] analysis has shown that all of the FDA-approved formulations can offer a 30% therapeutic gain over placebo.

Table 1 Adverse effects of mesalamine and thiopurines in a cohort of IBD patients[18]

Event	Number	Percentage (%)
Mesalamine (n = 44)		
Pulmonary dysfunction	3	6.8
Pancreatitis	1	2.2
Hemolytic anemia	1	2.2
Intolerance to local drug vehicle	1	2.2
Platelet reduction	1	2.2
Diarrhea	1	2.2
Total	8	17.8
Thiopurines (n = 57)		
Leukopenia	10	17.5
Hepatic damage	5	8.77
Infection	4	7.0
Pancreatitis	4	7.0
Idiosyncracy	2	3.50
Nausea	1	1.75
Malignancy	1	1.75
Total	27	47

Toxicity

Although they are prescribed worldwide, SASP/mesalamines can exert occasionally complex toxicity that targets the skin, kidneys, pancreas, liver and cardiovascular system. As a result of the sulfa moiety, SASP can target the skin more often with manifestations ranging from rashes to major Stevens-Johnson lesions. In contrast, the phenacetin-like structure confers on mesalamine the ability to effect necrosis of the renal papilla, thus explaining the concern for clinically meaningful renal toxicity. In an English survey[16], the frequency of 5-ASA-related interstitial nephritis was 11.1 cases per million prescriptions, with the figure being 7.5 for pancreatitis. The lung manifestations linked with mesalamine deserve particular attention[17], being probably based on allergic mechanisms shown to occasionally cause a range of lung damage from eosinophilic pneumonitis to bronchiolitis obliterans. In our series[18], an unexpected percentage of 6.8% patients out of 44 that received mesalamine showed respiratory distress or pleuro-pneumonitis (Table 1).

THIOPURINES

History

Belonging to the class of fraudulent nucleotides, the thiopurines are expected to exert a prevalent anti-proliferative and immunosuppressive effect by interfering with DNA replication and causing strand breakage. The thiopurines received a lot of attention in the 1950s and 60s from Gertrud Elion and George Hitchings, who aimed at exploiting their anti-proliferative actions for the treatment of pediatric malignancy. Some of the shrewd

perceptions of Elion, including the attempt to hasten the antineoplastic effects by adding the synergic drug allopurinol (see below), were already contained in her manuscripts of the 1960s[19], and such work led to her award of the Nobel prize in 1988[20]. In the following years, the oncological indications for thiopurines were challenged by the release of more potent antineoplastic drugs; However, an anecdotal claim at the beginning of the 1960s[21] opened the era of their use for IBD, an era that it still far from being concluded.

Metabolism and mechanism of action

6-mercaptopurine (6-MP), derived in the liver after non-enzymatic, glutathione-dependent, elaboration of the pro-drug azathioprine (AZA) opens the thiopurine metabolic cascade, which leads to the final synthesis of the immunosuppressive 6-thioguanine metabolites (6-TGNs). While AZA is mostly prescribed in Europe, 6-MP is preferred in the US. At the initial pathway, two enzymatic systems compete for the common substrate 6-MP: thiopurine methyltransferase (TPMT) catalyzes the formation of the methylated non-immunosuppressive compounds of methyl-mercaptopurine (MMP); and hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) leads to the synthesis of 6-thio-inosine-monophosphate. This metabolite may either enter a phosphorylation loop regulated by a pyrophosphatase (ITPase), or it may undergo elaboration by a dehydrogenase to form 6-TGN, which finally exerts their specific DNA strand breakage effect by incorporation into the DNA replication pathways (Figure 1). A few typical features of the toxicity of thiopurines can be accounted for by at least three points of genetic/biochemical variability, as contained in the described metabolic pathway. Also, such points have recently become the target for finely tuned interventions that are aimed at modulating the patterns of the metabolic cascade, and at restraining the clinical meaningfulness of the relevant thiopurine toxicity[22]. The three points include: the genetic polymorphism of TPMT; the possibility to inhibit the enzyme xanthine oxidase by allopurinol (Elion's initial idea); and the ITPase polymorphisms. The following paragraph on toxicity gives more insight into this matter.

Indications

The immunomodulatory action of thiopurines is characterized by a delayed onset that may take 3 mo. This feature, caused by some fine aspects of their immunomodulatory mechanisms as described below, has traditionally made the thiopurines specific remission maintenance drugs. A pivotal controlled study in 1980 has shown that 6-MP is superior to placebo for fistula closure and steroid sparing in CD[23]. Drug withdrawal experiments have shown a significant trend to relapse, with > 50% of the withdrawl patients relapsing at the third year of follow-up[24-26]. The efficacy of AZA in the maintenance of UC has been suggested by a similar withdrawal experiment conducted in 1992: out of 79 patients in remission on AZA and randomized to either continue or withdraw treatment, 36% of the former relapsed during follow-up, but as many as 59% of the latter relapsed[27]. Several multicenter and monocentric studies, including one from our group[28], have confirmed the efficacy of thiopurines in the maintenance of remission and avoidance of colectomy for UC. In particular, we were able to show that AZA is effective in the maintenance of fastidious forms of UC that are initially responsive to cyclosporin[29].

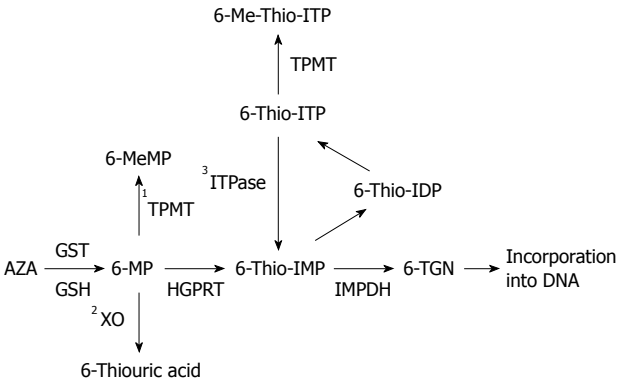

Figure 1 Thiopurine metabolism. For a caption to this figure please refer to metabolism and toxicity paragraphs and ref. 22. [1]TPMT is polymorphic and can be inhibited by mesalamine; [2]Xanthine oxidase can be inhibited by allopurinol; [3]ITPase is polymorphic.

Toxicity

Owing to the high degree of genetic polymorphism that affects a few of the key enzymes in their metabolic pathway, thiopurines tend to exert varied toxicity. A recent relevant review[30] includes the following: leukopenia 1.3%-12.6%; infection 0.3%-7.4%; liver damage 0%-4.2%; pancreatic damage 0%-4%; gastric intolerance 1.3%-6%; idiosyncratic reactions 0%-3.9%; and drug withdrawal 5.7%-22%. These figures have been upgraded in a recent review, which estimates that up to 40% of the subjects assigned to thiopurine treatment cannot benefit from these drugs because of the adverse effects[31], and do not differ from our monocentric experience[18] in which, of 57 IBD patients receiving thiopurines, 25 (43%) experienced adverse effects including leukopenia (17.5%), hepatic damage (8.7%), and infection (7.0%) (Table 1). Attempts at understanding and preventing leukopenia and hepatic and pancreatic events have brought about a significant increase in our basic knowledge. Examination of the thiopurine pathway suggests that leukopenia may chiefly be caused by dysfunction of the side methylator enzyme TPMT, which allows HGPRT to form excess levels of the bone-marrow-toxic 6-TGNs. Indeed, studies of TPMT activity have found it to be uneven, and the gene that encodes for TPMT has been shown to have a degree of polymorphism, as follows. Determination of erythrocyte TPMT activity has led to identification of three subsets of subjects: 90% show high activity; 10% show intermediate activity; and 0.3% are almost devoid of TPMT. The latter (1:300 in the Caucasian population) have a significant risk of developing fatal leukopenia if exposed to thiopurines. TPMT is encoded for by a highly polymorphic 27-kb long gene that is localized to chromosome 6p22.3. Seventeen

variant alleles have so far been described: three of these (TPMT*2, TPMT*3A, TPMT*3C) can cause the synthesis of an unstable protein, caused by an altered tertiary structure, and have been causally linked with 80%-95% of all cases of null TPMT activity. Such information in the last two decades has fostered the search for a screening test and has led to the standardization and release of commercial kits that are allegedly able to identify homozygous subjects at risk for developing fatal leukopenia. Such TPMT studies have provided the paradigm of the neonatal pharmacogenomic studies and have shown the extent of the clinical impact of these studies. If a hypoactive TPMT can cause leukopenia, recent studies[32] have suggested that hyperactivity of the enzyme may cause accumulation of an excess of methylated by-products and liver damage that may affect up to 10% of the so-called hypermethylator subjects. This problem has been addressed[31] by the co-prescription of allopurinol. By inhibiting xanthine oxidase and the production of the inactive thiouric acid, a 30% lower AZA dose can be administered, thus reducing the substrate for the hyperactive TPMT. Idiosyncratic pancreatitis has been claimed to respond to this strategy. In addition, idiosyncratic response to thiopurine, as shown by fever and flu-like syndromes, has been shown to be avoided by allopurinol. In these cases, probably the accumulation of ill-defined methylated toxins in the area of a polymorphic ITPase is the culprit. Although promising, research in the area of ITPase polymorphisms is still in its infancy.

CO-PRESCRIBED MESALAMINES AND THIOPURINES

Frequency in clinical practice

Data from a recent survey conducted by us and other four Italian centers has revealed a frequency of co-prescription of 71%.

Evidence of synergism between the two drugs

This comes essentially from withdrawal data in clinical practice. In two independent reports, others and ourselves have shown that patients on AZA and mesalamine in remission from their UC may relapse severely and progress to eventual colectomy if mesalamine is withdrawn; a consistent fall in the blood concentration of 6-TGNs is found in these circumstances[33,34]. In addition, the above cited review has shown that regular mesalamine therapy behaves as the only independent factor of continuous remission before AZA withdrawal.

Mechanisms underlying the synergy

As illustrated above (Figure 1), the purine metabolic pathway unfolds primarily following a process by which the pro-drug AZA is finally converted to the metabolites of 6-TGN. The immunosuppressive power of this biochemical machinery depends on the accumulation of the latter compounds, insofar as they are able to decrease the number of dividing lymphocytes by DNA strand breakage. The immunosuppressive steps of the pathway may be influenced by diversion of the metabolites towards two side-streams at the beginning of the process: one, catalyzed by TPMT, leads to the production of MMP, and the other, catalyzed by xanthine oxidase, produces thiouric acid, both of which are devoid of immunosuppressive activity. Of the two, TPMT has resulted in the most protean system, being under the influence of both genetic polymorphism and drugs. 5-ASA compounds have shown strong affinity for TPMT[35], with a significant inhibitory activity that results in increased feeding of metabolites towards the main axis, which results in a boost to the immunosuppressive power of the pathway. Mesalazine, sulphasalazine and olsalazine have all been shown to influence TPMT activity, with the effects emerging at both the biochemical and clinical levels, as detailed below.

Results from clinical reports

An increase of the effectiveness of AZA in relation to administration of mesalamine, and a concomitant increase in 6-TGN concentration, with attendant leukopenia, have been described in several studies[36-39]. Two other studies, on the contrary, have failed to find an advantage from co-prescription, whereas increased toxicity that has hastened the need to discontinue AZA has been emphasized[40,41]. The overall available evidence speaks in favor of co-prescribing AZA and 5-ASAs. Together with allopurinol, the mesalamines seem to offer an effective strategy to optimize AZA administration in hypermethylator patients. Whether this readily translates into improved clinical outcomes remains debatable. A recent systematic review[42], although not providing a definitive answer, has concluded that co-administration of thiopurines and mesalamines can lead to a decreased risk for colorectal cancer in long-standing disease.

REAPPRAISAL OF THE INDICATIONS FOR MESALAMINES: CHEMOPREVENTION

Background

Both CD and UC are known as pre-cancerous lesions, with the risk for transformation becoming significant within 8 years of the diagnosis of UC, and attaining 7%-14% at 25 years[43]. Two orders of evidence achieved in the last decades have focused attention on mesalamine as a chemopreventive agent against UC-dependent colorectal cancer. On one hand, a pivotal paper[1] has shown that unchecked inflammation acts independently in the promotion of cancer through dysplasia; and on the other hand, modern technology has provided evidence that mesalamine can exert a specific anti-neoplastic action thanks to its ability to interfere with both prostaglandins and nuclear transcription factors for the pro-inflammatory cytokines. The next question was whether mesalamine can protect against colon cancer *in vivo*. A literature search has retrieved at least three retrospective studies of correlation offering relevant answers: a 3-mo course of SASP significantly reduced

the cancer risk in a population of 3000 patients with colitis; in two subsets of colitis patients, of whom, only one received therapeutic doses of 5-ASA, the eventual percentage of cancer development was 3% *vs* 31%, respectively; and in the final study of 102 patients, the drug-induced risk reduction in cancer was 75%-90%[44].

Mechanisms

Peroxisome proliferator activated receptor gamma (PPAR-γ). PPAR-γ is a nuclear receptor that belongs to a family of at least 50 members that are involved with an array of biological functions. Once located to the nucleus and heterodimerized with retinoid X receptor alpha, PPAR-γ begins to regulate four gene classes. Such a gene complex is known to direct four major biological functions: metabolism, proliferation, signal transduction, and cell motility. PPAR-γ is maximally expressed in the gut, with a gradient increasing from the proximal to the distal bowel. A local negative gradient of expression has been shown in the colon, with the lowest expression levels found in the distal colon; microscopically, the expression is high among the proliferating cells of basal crypts and progressively fades away from bottom to top, to almost indistinguishable levels in cells that detach from the crypt apex and fall free in the lumen. This clearly depicts PPAR-γ as a potent differentiation factor that exerts its pivotal role in an environment where a dramatic proliferative drift completely renews the epithelium every 3 d.

These effects favor the candidacy of PPAR-γ to be identified among the effective antineoplastic agents in the colon. The insulin-sensitizing drugs, the glitazones, which bind PPAR-γ show anticancerous activity in animal models. Hemizygous knock-out animals for PPAR-γ show lesser resistance to carcinogenic treatments. At this point, the final crucial question is whether mesalamines can bind PPAR-γ, and two lines of evidence have contributed to the answer: (1) colitic animals that are heterozygous for PPAR-γ respond least to 5-ASA, which implies a role for PPAR-γ in the mediation of the antineoplastic effect of 5-ASA; and (2) 5-ASA can be accommodated into a loop in the structure of PPAR-γ through hydrogen bonding. Taken together, the above indicate that 5-ASA can exert a chemopreventive action against UC-related colorectal cancer, and this action is mediated through anti-inflammatory activity, and depends on its binding to a potent differentiation factor of colonocytes[45,46].

REAPPRAISAL OF THE POTENTIAL OF THIOPURINES: IMMUNOMODULATION

IBD patient caregivers have long become familiar with one of the hallmarks of the action of thiopurines: a latency of effect that may last for 3 mo. Traditionally, this delay has been attributed to the time supposed to be required for the 6-TGN metabolites to saturate myeloid precursors and exert their anti-proliferative effects, a tenet that has recently been challenged on the basis of two lines of evidence[47,48]: (1) the use of an intravenous load of AZA has not significantly reduced the latency; and (2) the process of myeloid cell saturation has been shown to be completed within 2 wk. The results of further studies prompted by these doubts have contributed to uncover that, well beyond their known anti-proliferative capacity, the thiopurines may exert a more finely tuned immunoregulatory action that is largely independent from DNA strand breakage and immune cell death. Focus on this novel aspect of their action is maintained by two recent publications.

Already back in 2003[49], the Neurath group had shown that thiopurines can induce T-cell apoptosis in controls as well as in IBD patients, by replacing GTP as a ligand for the Rac-1 receptor, thus hindering its main function of inducing NF-κB. This process is CD-28-dependent and triggers a mitochondrial pathway to apoptosis. This study was the first to demonstrate that a product of the intermediate thiopurine metabolism (6-thioguanine triphosphate, as generated from the phosphorylation loop described above) cannot simply break the native DNA, but specifically triggers apoptosis directed towards the autoimmune clones at the root of IBD perpetuation. This provides a fine immunological indication for the use of thiopurines, and hints at a disease-modifying role that is still to be studied.

The Ben-Horin group in Israel has recently developed research on this apoptosis process further, and has concentrated on timing and mechanistic issues[50]. Using a double *in vitro* and experimental approach in animals, they have gathered evidence that thiopurines effect a proliferative arrest of T lymphocytes, without any apoptotic effect being obvious until the fifth day post-stimulation. During this latency, these lingering T lymphocytes are still capable of adherence and mediating inflammation; thus, the immunological events lying behind the clinical latency of thiopurines have been uncovered. In their second set of experiments, this time conducted *in vivo*, they have shown that the animals must be exposed for > 1 mo to mercaptopurine before it restricts the memory pool to antigenic re-challenge. These data depict thiopurines as fine immunomodulators that require several weeks to express full-blown activity. Far from being speculative laboratory exercises, these approaches serve (1) to remind the clinician of the underlying reasons for the specific indication for thiopurines, as maintenance drugs; and (2) to remind doctors to reiterate to their patients the need for maximum compliance in order to maximally exploit the drug and benefit from the longest disease-free period.

CONCLUSION

Despite the time elapsed since their initial study, mesalamines and thiopurines continue to remain under investigation as research from basic immunology fosters novel clinical approaches, as shown by a few publications that have appeared even while preparing this review. Mesalamine has recently been proposed as the first candidate drug to be endowed with a disease-modifying role in UC[51]. Another review[52] has raised the question of

the timing of the introduction of AZA for IBD, and has proposed to use it earlier in a top-down strategy in order to best exploit its effects, chiefly the mucosal-healing potential. Finally, mesalamines and thiopurines, together with cyclosporin, are still considered unbeaten in terms of cost-effectiveness, when compared with the most recent drugs made by genetic engineering, and have been awarded the status of "backbone therapy" for IBD[53].

REFERENCES

1 **Rutter M**, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451-459

2 **Fiocchi C**, Ina K, Danese S, Leite AZ, Vogel JD. Alterations of mesenchymal and endothelial cells in inflammatory bowel diseases. *Adv Exp Med Biol* 2006; **579**: 168-176

3 **Svartz N**. Salazopyrin, a new sulfanilamide preparation. *Acta Medica Scand* 1942; **110**: 577-596

4 **Baron JH**, Connell AM, Lennard-Jones JE, Jones FA. Sulphasalazine and salicylazosulphadimidine in ulcerative colitis. *Lancet* 1962; **1**: 1094-1096

5 **Safdi AV**, Cohen RD. Review article: increasing the dose of oral mesalazine therapy for active ulcerative colitis does not improve remission rates. *Aliment Pharmacol Ther* 2007; **26**: 1179-1186

6 **Egan LJ**, Mays DC, Huntoon CJ, Bell MP, Pike MG, Sandborn WJ, Lipsky JJ, McKean DJ. Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *J Biol Chem* 1999; **274**: 26448-26453

7 **Dallegri F**, Ottonello L, Ballestrero A, Bogliolo F, Ferrando F, Patrone F. Cytoprotection against neutrophil derived hypochlorous acid: a potential mechanism for the therapeutic action of 5-aminosalicylic acid in ulcerative colitis. *Gut* 1990; **31**: 184-186

8 **Sandoval M**, Liu X, Mannick EE, Clark DA, Miller MJ. Peroxynitrite-induced apoptosis in human intestinal epithelial cells is attenuated by mesalamine. *Gastroenterology* 1997; **113**: 1480-1488

9 **Burress GC**, Musch MW, Jurivich DA, Welk J, Chang EB. Effects of mesalamine on the hsp72 stress response in rat IEC-18 intestinal epithelial cells. *Gastroenterology* 1997; **113**: 1474-1479

10 **Lauritsen K**, Laursen LS, Bukhave K, Rask-Madsen J. Effects of topical 5-aminosalicylic acid and prednisolone on prostaglandin E2 and leukotriene B4 levels determined by equilibrium in vivo dialysis of rectum in relapsing ulcerative colitis. *Gastroenterology* 1986; **91**: 837-844

11 **Ligumsky M**, Karmeli F, Sharon P, Zor U, Cohen F, Rachmilewitz D. Enhanced thromboxane A2 and prostacyclin production by cultured rectal mucosa in ulcerative colitis and its inhibition by steroids and sulfasalazine. *Gastroenterology* 1981; **81**: 444-449

12 **Malchow H**, Ewe K, Brandes JW, Goebell H, Ehms H, Sommer H, Jesdinsky H. European Cooperative Crohn's Disease Study (ECCDS): results of drug treatment. *Gastroenterology* 1984; **86**: 249-266

13 **Egan LJ**, Sandborn WJ. Advances in the treatment of Crohn's disease. *Gastroenterology* 2004; **126**: 1574-1581

14 **Hanauer S**, Schwartz J, Robinson M, Roufail W, Arora S, Cello J, Safdi M. Mesalamine capsules for treatment of active ulcerative colitis: results of a controlled trial. Pentasa Study Group. *Am J Gastroenterol* 1993; **88**: 1188-1197

15 **Sutherland L**, Macdonald JK. Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2006; CD000544

16 **Ransford RA**, Langman MJ. Sulphasalazine and mesalazine: serious adverse reactions re-evaluated on the basis of suspected adverse reaction reports to the Committee on Safety of Medicines. *Gut* 2002; **51**: 536-539

17 **Foster RA**, Zander DS, Mergo PJ, Valentine JF. Mesalamine-related lung disease: clinical, radiographic, and pathologic manifestations. *Inflamm Bowel Dis* 2003; **9**: 308-315

18 **Actis GC**, Pellicano R, Bugianesi E, Lagget M, Rizzetto M. Use of corticosteroids, immunomodulators, and infliximab at a third-level Day-Hospital Service of Gastro-Hepatology. *Minerva Gastroenterol Dietol* 2008; **54**: 239-242

19 **Elion GB**, Callahan S, Rundles RW, Hitchings GH. Relationship between metabolic fates and antitumor activities of thiopurines. *Cancer Res* 1963; **23**: 1207-1217

20 **Autobiography of Gertrude B**. Elion, the Nobel Prize in Physiology or Medicine 1988. *Oncologist* 2006; **11**: 966-968

21 **Bean RH**. The treatment of chronic ulcerative colitis with 6-mercaptopurine. *Med J Aust* 1962; **49**(2): 592-593

22 **Sahasranaman S**, Howard D, Roy S. Clinical pharmacology and pharmacogenetics of thiopurines. *Eur J Clin Pharmacol* 2008; **64**: 753-767

23 **Present DH**, Korelitz BI, Wisch N, Glass JL, Sachar DB, Pasternack BS. Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N Engl J Med* 1980; **302**: 981-987

24 **Bouhnik Y**, Lemann M, Mary JY, Scemama G, Tai R, Matuchansky C, Modigliani R, Rambaud JC. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215-219

25 **Lemann M**, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Modigliani R, Bouhnik Y. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812-1818

26 **Treton X**, Bouhnik Y, Mary JY, Colombel JF, Duclos B, Soule JC, Lerebours E, Cosnes J, Lemann M. Azathioprine withdrawal in patients with Crohn's disease maintained on prolonged remission: a high risk of relapse. *Clin Gastroenterol Hepatol* 2009; **7**: 80-85

27 **Hawthorne AB**, Logan RF, Hawkey CJ, Foster PN, Axon AT, Swarbrick ET, Scott BB, Lennard-Jones JE. Randomised controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ* 1992; **305**: 20-22

28 **Ardizzone S**, Cassinotti A, Actis GC, Duca P, D'Albasio G, Gai E, Massari A, Bosani M, Colombo E, Manes G, Maconi G, Bianchi-Porro G. Maintenance treatment with azathioprine in ulcerative colitis:outcomes after drug withdrawal in patients with sustained remission. *Gastroenterology* 2007; **132**(4): S1138

29 **Actis GC**, Fadda M, David E, Sapino A. Colectomy rate in steroid-refractory colitis initially responsive to cyclosporin: a long-term retrospective cohort study. *BMC Gastroenterol* 2007; **7**: 13

30 **Siegel CA**, Sands BE. Review article: practical management of inflammatory bowel disease patients taking immunomodulators. *Aliment Pharmacol Ther* 2005; **22**: 1-16

31 **Ansari A**, Elliott T, Baburajan B, Mayhead P, O'Donohue J, Chocair P, Sanderson J, Duley J. Long term outcome of using allopurinol co-therapy as a strategy for overcoming thiopurine hepatotoxicity in treating inflammatory bowel disease. *Aliment Pharmacol Ther* 2008; Epub ahead of print

32 **Bastida G**, Nos P, Aguas M, Beltran B, Rubin A, Dasi F, Ponce J. Incidence, risk factors and clinical course of thiopurine-induced liver injury in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2005; **22**: 775-782

33 **Actis GC**, Marzano A, Pellicano R, Rizzetto M. How important is mesalamine in the maintenance of steroid-refractory colitis? *Inflamm Bowel Dis* 2008; **14**: 1026

34 **Stocco G**, Martelossi S, Malusa' N, Marino S, Decorti G, Bartoli F, Ventura A. Interruption of mesalamine and reduction of the blood concentration of the active metabolites of azathioprine: possible causes of ulcerative colitis relapse. *Dig Dis Sci* 2008; **53**: 3246-3249

35 **Lewis LD**, Benin A, Szumlanski CL, Otterness DM, Lennard

L, Weinshilboum RM, Nierenberg DW. Olsalazine and 6-mercaptopurine-related bone marrow suppression: a possible drug-drug interaction. *Clin Pharmacol Ther* 1997; **62**: 464-475

36 **de Boer NK**, Wong DR, Jharap B, de Graaf P, Hooymans PM, Mulder CJ, Rijmen F, Engels LG, van Bodegraven AA. Dose-dependent influence of 5-aminosalicylates on thiopurine metabolism. *Am J Gastroenterol* 2007; **102**: 2747-2753

37 **Lowry PW**, Franklin CL, Weaver AL, Pike MG, Mays DC, Tremaine WJ, Lipsky JJ, Sandborn WJ. Measurement of thiopurine methyltransferase activity and azathioprine metabolites in patients with inflammatory bowel disease. *Gut* 2001; **49**: 665-670

38 **Hande S**, Wilson-Rich N, Bousvaros A, Zholudev A, Maurer R, Banks P, Makrauer F, Reddy S, Burakoff R, Friedman S. 5-aminosalicylate therapy is associated with higher 6-thioguanine levels in adults and children with inflammatory bowel disease in remission on 6-mercaptopurine or azathioprine. *Inflamm Bowel Dis* 2006; **12**: 251-257

39 **Dewit O**, Vanheuverzwyn R, Desager JP, Horsmans Y. Interaction between azathioprine and aminosalicylates: an in vivo study in patients with Crohn's disease. *Aliment Pharmacol Ther* 2002; **16**: 79-85

40 **Mantzaris GJ**, Sfakianakis M, Archavlis E, Petraki K, Christidou A, Karagiannidis A, Triadaphyllou G. A prospective randomized observer-blind 2-year trial of azathioprine monotherapy versus azathioprine and olsalazine for the maintenance of remission of steroid-dependent ulcerative colitis. *Am J Gastroenterol* 2004; **99**: 1122-1128

41 **Shah JA**, Edwards CM, Probert CS. Should azathioprine and 5-aminosalicylates be coprescribed in inflammatory bowel disease?: an audit of adverse events and outcome. *Eur J Gastroenterol Hepatol* 2008; **20**: 169-173

42 **Andrews JM**, Travis SP, Gibson PR, Gasche C. Systematic review: does concurrent therapy with 5-ASA and immunomodulators in inflammatory bowel disease improve outcomes? *Aliment Pharmacol Ther* 2009; **29**: 459-469

43 **Bernstein CN**. Cancer prevention strategies in inflammatory bowel disease. In: Bayless T, Hanauer SB, editors. Advanced therapy of inflammatory bowel disease. London: BC Decker Inc, 2001: 257-261

44 **Actis GC**, Pazienza P, Rosina F. Mesalamine for inflammatory bowel disease: recent reappraisals. *Inflamm Allergy Drug Targets* 2008; **7**: 1-5

45 **Thompson EA**. PPARgamma physiology and pathology in gastrointestinal epithelial cells. *Mol Cells* 2007; **24**: 167-176

46 **Dubuquoy L**, Rousseaux C, Thuru X, Peyrin-Biroulet L, Romano O, Chavatte P, Chamaillard M, Desreumaux P. PPARgamma as a new therapeutic target in inflammatory bowel diseases. *Gut* 2006; **55**: 1341-1349

47 **Balis FM**, Holcenberg JS, Poplack DG, Ge J, Sather HN, Murphy RF, Ames MM, Waskerwitz MJ, Tubergen DG, Zimm S, Gilchrist GS, Bleyer WA. Pharmacokinetics and pharmacodynamics of oral methotrexate and mercaptopurine in children with lower risk acute lymphoblastic leukemia: a joint children's cancer group and pediatric oncology branch study. *Blood* 1998; **92**: 3569-3577

48 **Sandborn WJ**, Tremaine WJ, Wolf DC, Targan SR, Sninsky CA, Sutherland LR, Hanauer SB, McDonald JW, Feagan BG, Fedorak RN, Isaacs KL, Pike MG, Mays DC, Lipsky JJ, Gordon S, Kleoudis CS, Murdock RH Jr. Lack of effect of intravenous administration on time to respond to azathioprine for steroid-treated Crohn's disease. North American Azathioprine Study Group. *Gastroenterology* 1999; **117**: 527-535

49 **Tiede I**, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R, Walczak H, Iven H, Galle PR, Ahmadian MR, Neurath MF. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* 2003; **111**: 1133-1145

50 **Ben-Horin S**, Goldstein I, Fudim E, Picard O, Yerushalmi Z, Barshack I, Bank I, Goldschmid Y, Meir SB, Mayer L, Chowers Y. Early preservation of effector functions followed by eventual T cell memory depletion: a model for the delayed onset of the effect of thiopurines. *Gut* 2009; **58**: 396-403

51 **Hanauer SB**. Review article: evolving concepts in treatment and disease modification in ulcerative colitis. *Aliment Pharmacol Ther* 2008; **27** Suppl 1: 15-21

52 **Etchevers MJ**, Aceituno M, Sans M. Are we giving azathioprine too late? The case for early immunomodulation in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 5512-5518

53 **Schwartz M**, Cohen R. Optimizing conventional therapy for inflammatory bowel disease. *Curr Gastroenterol Rep* 2008; **10**: 585-590

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REVIEW

Acute pancreatitis: Etiology and common pathogenesis

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Abstract

Acute pancreatitis is an inflammatory disease of the pancreas. The etiology and pathogenesis of acute pancreatitis have been intensively investigated for centuries worldwide. Many causes of acute pancreatitis have been discovered, but the pathogenetic theories are controversial. The most common cause of acute pancreatitis is gallstone impacting the distal common bile-pancreatic duct. The majority of investigators accept that the main factors for acute billiary pancreatitis are pancreatic hyperstimulation and bile-pancreatic duct obstruction which increase pancreatic duct pressure and active trypsin reflux. Acute pancreatitis occurs when intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed. However, little is known about the other acute pancreatitis. We hypothesize that acute biliary pancreatitis and other causes of acute pancreatitis possess a common pathogenesis. Pancreatic hyperstimulation and pancreatic duct obstruction increase pancreatic duct pressure, active trypsin reflux, and subsequent unregulated activation of trypsin within pancreatic acinar cells. Enzyme activation within the pancreas leads to auto-digestion of the gland and local inflammation. Once the hypothesis is confirmed, traditional therapeutic strategies against acute pancreatitis may be improved. Decompression of pancreatic duct pressure should be advocated in the treatment of acute pancreatitits which may greatly improve its outcome.

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Key words: Acute pancreatitis; Pathogenesis; Etiology; Pancreatic duct obstruction; Pancreatic hyperstimulation; Pancreatic duct pressure

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INTRODUCTION

Acute pancreatitis, an inflammatory disease of the pancreas, is mild and resolves itself without serious complications in 80% of patients, but it has complications and a substantial mortality in up to 20% of patients[1]. Its etiology and pathogenesis have been intensively investigated for centuries worldwide[2]. In 1856, Claude Bernard suggested that bile reflux into the common pancreatic duct could trigger acute pancreatitis[3]. Several subsequent studies led to theories fuelling the debate until 1901[4], when Eugene Opie proposed that gallstone migration into the common bile duct is the main cause of acute pancreatitis[5]. Since then, many other causes of pancreatitis have been discovered[6]. However, the pathogenesis of acute pancreatitis is still controversial to date. Several theories attempt to explain the pathogenesis of acute pancreatitis. In terms of disease pathogenesis, whether acute pancreatitis is really one entity or it comprises a group of distinct pathogenic entities remains unclear. From a pathogenic perspective, acute pancreatitis is an identity crisis[7].

CAUSES OF ACUTE PANCREATITIS

There are many causes of acute pancreatitis, which can be easily identified in 75%-85% of patients. In developed countries, obstruction of the common bile duct by stones (38%) and alcohol abuse (36%) are the most frequent causes of acute pancreatitis[3,8]. Gallstone-induced pancreatitis is caused by duct obstruction by gallstone migration. Obstruction is localized in the bile duct and pancreatic duct, or both. Duct obstruction promotes pancreatitis by increasing duct pressure and subsequent unregulated activation of digestive

enzymes[9]. Alcohol abuse is the second most frequent cause of acute pancreatitis, but the correlation between alcohol and pancreatitis is not completely understood[10]. In experimental models, Gorelick showed that ethanol directly sensitizes acinar cells to cholecystokinin stimulation. As the development of pancreatitis might be affected by both genetic and environmental factors, failure to inhibit trypsin activity or to wash active trypsin into pancreatic ducts might promote alcoholic pancreatitis[11]. In fact, the exact mechanism underlying alcoholic acute pancteatitis has not been extensively elucidated.

Pancreas divisum, a common congenital anatomical variant of the pancreatic duct in about 7% of autopsy series, results from the absence of fusion between the dorsal and ventral ductal systems. The possible consequence of pancreas divisum is a stenosed or inadequately patent minor papilla, preventing normal drainage of pancreatic secretions and leading to increased intraductal pressure. However, whether pancreas divisum is related to pancreatitis is highly controversial[12]. Whether dysfunction of sphincter of Oddi can trigger acute pancreatitis by increasing intrapancreatic duct pressure is also controversial[13]. Biliary sludge refers to a viscous bile suspension that contains cholesterol crystals and calcium bilirubinate granules embedded in strands of gallbladder mucus. Sludge is associated with bile stasis, long-lasting fast, distal bile duct obstruction, and total parenteral feeding. Most patients with biliary sludge are asymptomatic. Biliary sludge is commonly seen in patients with recurrent acute pancreatitis of unknown origin, and cholecystectomy might prevent the recurrence of pancreatic disease[14].

Intraduct papillary mucinous tumor might be another cause of acute pancreatitis. Tumor or mucus produced by it obstructs the main pancreatic duct and its side branch, or both. Logically, the consequence is increased pancreatic duct pressure caused by pancreatic hyperstimulation and pancreatic duct obstruction. Thus, these tumors might trigger acute pancreatitis through the same mechanisms underlying acute biliary pancreatitis[15].

Endoscopic retrograde cholangiopancreatography (ERCP) is a potential cause of acute pancreatitis. Asymptomatic hyperamylasaemia occurs in 35%-70% of patients after the procedure. ERCP has a higher risk of inducing acute pancreatitis when it is performed to treat Oddi sphincter dysfunction than to remove gallstones in the bile duct. Other risk factors for post-ERCP pancreatitis include young age, female sex, number of attempts to cannulate papilla, and poor emptying of pancreatic duct after opacification. Prevention of post-ERCP pancreatitis in high-risk patients might be achieved by placing a temporary pancreatic stent[16].

Hypercalcaemia is another rare and inconsistent cause of acute pancreatitis. Because the incidence of pancreatitis is low in patients with chronic hypercalcaemia, additional factors are probably needed to induce pancreatitis[17]. Drugs rarely induce acute pancreatitis. Cases of drug-induced pancreatitis have been reported[18]. Many infectious agents are associated with acute pancreatitis, but no microorganism has ever been identified within the pancreas. However, it was reported that acute pancreatitis is associated with viral or bacterial infections, and infestation with parasites[19]. Although a few researchers speculated that unexplained recurrent acute pancreatitis might be associated with some known genetic mutations, no decisive and persuasive evidence supports the notion[20].

In summary, many causes of acute pancreatitis have been discovered. The main causes are gallstone migration and alcohol abuse. Other causes are uncommon, situational, or controversial. Although there are many theories about the pathogenesis of acute pancreatitis, they are still controversial. These causes have not yet been completely elucidated.

MULTIPLE AND CONTROVERSIAL PATHOGENETIC THEORIES

For centuries, the pathogenesis of acute pancreatitis has been intensively investigated worldwide[2]. Many theories have been proposed attempting to explain the pathogenetic mechanisms underlying acute pancreatitis[21]. The important theories about the pathogenesis of acute pancreatitis include bile-pancreatic duct common pathway theory, pancreatic autodigestion theory, gallstone migration theory, enzyme activation theory, kinin and complement system activation theory, microcirculation disturbance theory, leukocyte excessive activation theory, pancreatic acinar cell apoptosis and necrosis theory, all of which are still controversial[22]. They can only explain the pathogenesis of some specific pancreatitis cases, or specific aspects of pathogenetic process of some forms of acute pancreatitis. In fact, no ideal theories on the pathogenesis of acute pancreatitis are available at present.

Although 70%-80% of acute pancreatitis cases are due to alcohol abuse and gallstones, the exact mechanisms by which they initiate acute pancreatitis are unknown. In addition, because of its rapid course and the relative inaccessibility of pancreatic tissue for examination during pancreatitis, investigations of the mechanisms underlying these pathobiologic processes have been hampered. Considering these obstacles, investigators have turned to animal models of acute pancreatitis to reveal the molecular steps initiating these pathobiologic responses to identify potential targets for therapeutic intervention[23-25]. Although the exact mechanisms underlying acute pancreatitis caused by alcohol and gallstones in humans have not been established, key steps in mediating the pathobiologic processes that define acute pancreatitis can be identified from animal models, and used to develop therapies that can be ultimately tested in human pancreatitis[26].

Since early in the twentieth century, a good many of experimental studies based on animal models have been carried out[27]. Many research results support that bile reflux and pancreatic auto-digestion by trypsin are central to the pathogenesis of gallstone pancreatitis[28]. A century later, the following questions still remain to be

answered: whether it is rational to examine the possibility that gallstone pancreatitis develops without reflux of bile into the pancreatic duct, whether trypsinogen activation is an effect rather than the cause of pancreatitis, whether active trypsin is essential for the development of acute pancreatitis or whether it is merely a secondary factor that exacerbates pancreatitis. It has been shown that bile reflux is not a necessary factor for acute pancreatitis[29]. In opossum, merely ligation of the pancreatic duct can cause necrotizing acute pancreatitis[30], but in rats or in rabbits, this causes apoptosis and atrophy of pancreas[31], suggesting that further study is needed to elucidate the pathogenesis of acute panceatitis in order to explain the paradoxical experimental results with different animals.

Considering various causes of acute pancreatitis, the question of whether each cause of acute pancreatitis corresponds to specific pathogenesis or various causes of acute pancreatitis actually possess a common pathogenesis should be answered. We hypothesize that, irrespective of the etiology of acute pancreatitis, there is a common pathway that triggers various forms of acute pancreatitis.

COMMON PATHOGENESIS AND VARIOUS CAUSES OF ACUTE PANCREATITIS

The etiology and pathogenesis of acute pancreatitis have been intensively investigated[2], but the pathogenetic theories are controversial. The predominant theories of acute biliary pancreatitis are common pathway theory and gallstone migration theory, which consent that the key factor for acute biliary pancreatitis is bile-pancreatic duct obstruction, which increases pancreatic duct pressure, bile reflux, trypsin activation and pancreatic auto-digestion[32]. Acute pancreatitis occurs when intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed[33]. However, these theories are controversial.

Although pancreatic duct obstruction may play an important role in the pathogenesis of gallstone pancreatitis, it is not sufficient to cause the morphological changes of acute pancreatitis[34], indicating that other events must occur if the changes induced by pancreatic duct obstruction lead to acute pancreatitis. Although acinar hyperstimulation has often been implicated in acute pancreatitis pathogenesis, there is no evidence that supports it[35]. We hypothesize that pancreatic acinar hyperstimulation, in the presence of duct obstruction, triggers and exacerbate acute pancreatitis.

We speculate that the main preconditions that trigger acute biliary pancreatitis are pancreatic hyperstimulation and bile-pancreatic duct obstruction, which increase pancreatic duct pressure, active trypsin reflux, and unregulated activation of trypsin within pancreatic acinar cells. Enzyme activation within the pancreas leads to auto-digestion of the gland and local inflammation. However, little is known about the other causes of acute pancreatitis.

We hypothesize that there is a common pathogenic pathway that triggers various forms of acute pancreatitis: acute biliary pancreatitis and other forms of acute pancreatitis. In our hypothesis, there are various causes which may cause acute pancreatitis and lead to pancreatic duct obstruction and blockage of pancreatic juice outflow under certain circumstances. In the presence of exocrine pancreatic hyperstimulation, pancreatic duct pressure, active trypsin reflux, and unregulated activation of trypsin within pancreatic canard cells would increase. When intracellular protective mechanisms to prevent trypsinogen activation or reduce trypsin activity are overwhelmed, acute pancreatitis occurs.

CONCLUSION

Acute pancreatitis has been intensively studied for centuries. Many causes of acute pancreatitis have been discovered, but its pathogenetic theories are multiple and controversial. The true nature of acute pancreatitis still remains to be elucidated. The causes of acute pancreatitis are various, and its mechanism is common. Once the hypothesis is confirmed, traditional therapeutic strategies against acute pancreatitis may be improved, and decompression of pancreatic duct pressure should be advocated in the treatment of acute pancreatitits which may greatly improve the outcome of acute pancreatitis[36,37].

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REFERENCES

1 **Lund H**, Tønnesen H, Tønnesen MH, Olsen O. Long-term recurrence and death rates after acute pancreatitis. *Scand J Gastroenterol* 2006; **41**: 234-238
2 **Pandol SJ**. Acute pancreatitis. *Curr Opin Gastroenterol* 2006; **22**: 481-486
3 **Lankisch PG**, Assmus C, Lehnick D, Maisonneuve P, Lowenfels AB. Acute pancreatitis: does gender matter? *Dig Dis Sci* 2001; **46**: 2470-2474
4 **Eichelberger MR**, Chatten J, Bruce DA, Garcia VF, Goldman M, Koop CE. Acute pancreatitis and increased intracranial pressure. *J Pediatr Surg* 1981; **16**: 562-570
5 **Steer LM**. Etiology and pathogenesis of acute pancreatitis. *Ann Ital Chir* 1995; **66**: 159-163
6 **Frossard JL**, Steer ML, Pastor CM. Acute pancreatitis. *Lancet* 2008; **371**: 143-152
7 **Samuel I**. Bile and pancreatic juice exclusion activates acinar stress kinases and exacerbates gallstone pancreatitis. *Surgery* 2008; **143**: 434-440
8 **Spanier BW**, Dijkgraaf MG, Bruno MJ. Epidemiology, aetiology and outcome of acute and chronic pancreatitis: An update. *Best Pract Res Clin Gastroenterol* 2008; **22**: 45-63
9 **Diehl AK**, Holleman DR Jr, Chapman JB, Schwesinger WH, Kurtin WE. Gallstone size and risk of pancreatitis. *Arch Intern Med* 1997; **157**: 1674-1678
10 **Gorelick FS**. Alcohol and zymogen activation in the pancreatic acinar cell. *Pancreas* 2003; **27**: 305-310
11 **Whitcomb DC**. Genetic polymorphisms in alcoholic pancreatitis. *Dig Dis* 2005; **23**: 247-254

12 **Gelrud A**, Sheth S, Banerjee S, Weed D, Shea J, Chuttani R, Howell DA, Telford JJ, Carr-Locke DL, Regan MM, Ellis L, Durie PR, Freedman SD. Analysis of cystic fibrosis gener product (CFTR) function in patients with pancreas divisum and recurrent acute pancreatitis. *Am J Gastroenterol* 2004; **99**: 1557-1562

13 **Fazel A**, Geenen JE, MoezArdalan K, Catalano MF. Intrapancreatic ductal pressure in sphincter of Oddi dysfunction. *Pancreas* 2005; **30**: 359-362

14 **Lee SP**, Nicholls JF, Park HZ. Biliary sludge as a cause of acute pancreatitis. *N Engl J Med* 1992; **326**: 589-593

15 **Pilleul F**, Rochette A, Partensky C, Scoazec JY, Bernard P, Valette PJ. Preoperative evaluation of intraductal papillary mucinous tumors performed by pancreatic magnetic resonance imaging and correlated with surgical and histopathologic findings. *J Magn Reson Imaging* 2005; **21**: 237-244

16 **Cheng CL**, Sherman S, Watkins JL, Barnett J, Freeman M, Geenen J, Ryan M, Parker H, Frakes JT, Fogel EL, Silverman WB, Dua KS, Aliperti G, Yakshe P, Uzer M, Jones W, Goff J, Lazzell-Pannell L, Rashdan A, Temkit M, Lehman GA. Risk factors for post-ERCP pancreatitis: a prospective multicenter study. *Am J Gastroenterol* 2006; **101**: 139-147

17 **Bess MA**, Edis AJ, van Heerden JA. Hyperparathyroidism and pancreatitis. Chance or a causal association? *JAMA* 1980; **243**: 246-247

18 **Lankisch PG**, Dröge M, Gottesleben F. Drug induced acute pancreatitis: incidence and severity. *Gut* 1995; **37**: 565-567

19 **Parenti DM**, Steinberg W, Kang P. Infectious causes of acute pancreatitis. *Pancreas* 1996; **13**: 356-371

20 **Felley C**, Morris MA, Wonkam A, Hirschel B, Flepp M, Wolf K, Furrer H, Battegay M, Bernasconi E, Telenti A, Frossard JL. The role of CFTR and SPINK-1 mutations in pancreatic disorders in HIV-positive patients: a case-control study. *AIDS* 2004; **18**: 1521-1527

21 **Gabryelewicz A**. Etiology and pathogenesis of acute pancreatitis--current view. *Rocz Akad Med Bialymst* 1995; **40**: 218-226

22 **Yadav D**, Lowenfels AB. Trends in the epidemiology of the first attack of acute pancreatitis: a systematic review. *Pancreas* 2006; **33**: 323-330

23 **Laukkarinen JM**, Weiss ER, van Acker GJ, Steer ML, Perides G. Protease-activated receptor-2 exerts contrasting model-specific effects on acute experimental pancreatitis. *J Biol Chem* 2008; **283**: 20703-20712

24 **Samuel I**, Tephly L, Williard DE, Carter AB. Enteral exclusion increases MAP kinase activation and cytokine production in a model of gallstone pancreatitis. *Pancreatology* 2008; **8**: 6-14

25 **Yang F**, Wang Y, Sternfeld L, Rodriguez JA, Ross C, Hayden MR, Carriere F, Liu G, Schulz I. The role of free fatty acids, pancreatic lipase and Ca+ signalling in injury of isolated acinar cells and pancreatitis model in lipoprotein lipase-deficient mice. *Acta Physiol (Oxf)* 2009; **195**: 13-28

26 **Pandol SJ**, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. *Gastroenterology* 2007; **133**: 1056.e1-1056.e25

27 **Foitzik T**, Hotz HG, Eibl G, Buhr HJ. Experimental models of acute pancreatitis: are they suitable for evaluating therapy? *Int J Colorectal Dis* 2000; **15**: 127-135

28 **Chen JW**, Thomas A, Woods CM, Schloithe AC, Toouli J, Saccone GT. Sphincter of Oddi dysfunction produces acute pancreatitis in the possum. *Gut* 2000; **47**: 539-545

29 **Arendt T**, Nizze H, Mönig H, Kloehn S, Stüber E, Fölsch UR. Biliary pancreatic reflux-induced acute pancreatitis--myth or possibility? *Eur J Gastroenterol Hepatol* 1999; **11**: 329-335

30 **Ohshio G**, Saluja A, Steer ML. Effects of short-term pancreatic duct obstruction in rats. *Gastroenterology* 1991; **100**: 196-202

31 **Lerch MM**, Saluja AK, Rünzi M, Dawra R, Saluja M, Steer ML. Pancreatic duct obstruction triggers acute necrotizing pancreatitis in the opossum. *Gastroenterology* 1993; **104**: 853-861

32 **Saluja A**, Saluja M, Villa A, Leli U, Rutledge P, Meldolesi J, Steer M. Pancreatic duct obstruction in rabbits causes digestive zymogen and lysosomal enzyme colocalization. *J Clin Invest* 1989; **84**: 1260-1266

33 **Armstrong CP**, Taylor TV, Torrance HB. Pressure, volume and the pancreas. *Gut* 1985; **26**: 615-624

34 **Meyerholz DK**, Samuel I. Morphologic characterization of early ligation-induced acute pancreatitis in rats. *Am J Surg* 2007; **194**: 652-658

35 **Samuel I**, Toriumi Y, Zaheer A, Joehl RJ. Mechanism of acute pancreatitis exacerbation by enteral bile-pancreatic juice exclusion. *Pancreatology* 2004; **4**: 527-532

36 **Uomo G**, Slavin J. Endoscopic sphincterotomy for acute pancreatitis: arguments in favour. *Ital J Gastroenterol Hepatol* 1998; **30**: 557-561

37 **Lucas CE**, McIntosh B, Paley D, Ledgerwood AM, Vlahos A. Surgical decompression of ductal obstruction in patients with chronic pancreatitis. *Surgery* 1999; **126**: 790-795; discussion 795-797

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ORIGINAL ARTICLES

Transforming growth factor-β1 induces intestinal myofibroblast differentiation and modulates their migration

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Abstract

AIM: To investigate the effects of transforming growth factor β1 (TGF-β1) on the differentiation of colonic lamina propria fibroblasts (CLPF) into myofibroblasts *in vitro*.

METHODS: Primary CLPF cultures were incubated with TGF-β1 and analyzed for production of α-smooth muscle actin (α-SMA), fibronectin (FN) and FN isoforms. Migration assays were performed in a modified 48-well Boyden chamber. Levels of total and phosphorylated focal adhesion kinase (FAK) in CLPF were analyzed after induction of migration.

RESULTS: Incubation of CLPF with TGF-β1 for 2 d did not change α-SMA levels, while TGF-β1 treatment for 6 d significantly increased α-SMA production. Short term incubation (6 h) with TGF-β1 enhanced CLPF migration, while long term treatment (6 d) of CLPF with TGF-β1 reduced migration to 15%-37% compared to untreated cells. FN and FN isoform mRNA expression were increased after short term incubation with TGF-β1 (2 d) in contrast to long term incubation with TGF-β1 for 6 d. After induction of migration, TGF-β1-preincubated CLPF showed higher amounts of FN and its isoforms and lower levels of total and phosphorylated FAK than untreated cells.

CONCLUSION: Long term incubation of CLPF with TGF-β1 induced differentiation into myofibroblasts with enhanced α-SMA, reduced migratory potential and FAK phosphorylation, and increased FN production. In contrast, short term contact (6 h) of fibroblasts with TGF-β1 induced a dose-dependent increase of cell migration and FAK phosphorylation without induction of α-SMA production.

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Key words: Transforming growth factor β1; Colonic fibroblasts; Myofibroblasts; Migration; Fibronectin

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INTRODUCTION

Transforming growth factor-β (TGF-β) is involved in multiple fundamental biological processes such as cell proliferation[1-3], cell migration[4-8], cell differentiation[9], extracellular matrix deposition[6,7,10,11], and immune responses[12]. TGF-β is secreted by a variety of cells

including platelets, monocytes, macrophages, and lymphocytes. It is thought to play a critical role in embryogenesis, host response to tumors, and the repair response that follows damage to tissues by immune and nonimmune reactions[8].

TGF-β not only affects remodeling during normal wound healing after tissue injury, but also enhances fibrogenesis. Therefore, it is an important mediator in multiple fibrotic diseases, including pulmonary fibrosis, liver fibrosis, chronic pancreatitis[12], and in stricture formation in Crohn's disease (CD)[13]. Increased expression of TGF-β, especially of the isoform TGF-β1, has been demonstrated in fibrotic tissues and regions of increased extracellular matrix deposition[12]. TGF-β changes the balance between extracellular matrix synthesis and degradation, inducing an increase in the synthesis of matrix components and a parallel decrease in the matrix-proteolytic activity[11,12].

TGF-β is synthesized as a pro-form which can be activated by multiple mechanisms. After activation of latent TGF-β and binding to its receptors, the activated receptors phosphorylate and assemble cytoplasmic Smad proteins. Subsequently, Smad complexes move to the nucleus as transcriptional regulators[12,14].

TGF-β is also a potent chemoattractant for human dermal fibroblasts. Intact disulfide bonds and perhaps the dimeric structure of TGF-β are essential for its ability to stimulate migration of fibroblasts, since reduction of TGF-β results in a marked loss of its chemoattractant potency[8].

Fibroblast migration plays an important role in tissue formation and wound healing. Following injury, tissue repair takes place involving inflammation, new tissue formation and scar constitution[15]. Migration of fibroblasts into and through the extracellular matrix during the initial phase of wound healing appears to be a fundamental component of wound contraction. In recent studies, we found that colonic lamina propria fibroblasts (CLPF) conditioned media induce migration of primary human CLPF in the modified Boyden chamber[16]. Furthermore, we demonstrated that fibronectin (FN) was mainly responsible for the autocrine induction of CLPF migration and was an essential requirement for the induction of CLPF migration, since different growth factors enhanced CLPF migration only in the presence of conditioned medium or recombinant FN[16]. Reduced and enhanced migratory potential of CLPF correlated with decreased and increased amounts of FN or FN isoforms, respectively[17]. FNs occur in up to 20 different isoforms as a result of alternative splicing of the primary transcript in the two homologous type Ⅲ domains named ED-A and ED-B and the one non-homologous repeat termed ⅢCS[18-22]. The ED-A and ED-B segments are either entirely included or excluded. The type Ⅲ repeat may be included, excluded, or partially included in FN[23-25].

The alternatively spliced FN isoforms show distinct functional differences. The expression of FN containing ED-A and ED-B domains is significantly increased during physiological wound healing and pathological tissue fibrosis[26]. Changes in the migratory potential of CLPF from patients with CD were associated with changes in FN isoform level, while the expression of integrin α5β1, the main FN receptor on the surface of CLPF, was unchanged[17].

The differentiation or activation of fibroblasts into myofibroblasts is an important step in tissue repair. Transient appearance of myofibroblasts is a feature of normal wound healing, but the persistence of these activated cells is associated with excessive collagen deposition and fibrosis[9]. Their prolonged presence and over-representation are hallmarks in the pathophysiology of tissue fibrosis[27]. TGF-β1 potently stimulates the production of α-smooth muscle actin (α-SMA) and stress fiber formation in fibroblasts and therefore their differentiation into myofibroblasts[9].

Since the regulation of migration and differentiation of intestinal fibroblasts is an important mechanism during intestinal wound healing and fibrosis, the effect of TGF-β1 on these processes and on FN and FN isoform production was investigated in this study.

MATERIALS AND METHODS

Patients

Primary CLPF cultures were obtained from endoscopic biopsies or surgical specimens taken from healthy areas of the mucosa of patients undergoing surveillance colonoscopy or surgery for colorectal carcinoma. The study was approved by the Ethics Committee of the University of Regensburg.

Isolation and culture of human colonic fibroblasts

Human CLPF were isolated and cultured as described earlier[28]. Briefly, mucosa from surgical specimens was cut into 1 mm pieces while the biopsies were used directly for the isolation of CLPF. Epithelial cells were removed in Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+} (PAA, Cölbe, Germany) with 2 mmol/L EDTA (SIGMA, Deisenhofen, Germany). The remaining tissue was rinsed and then digested for 30 min at 37℃ with 1 mg/mL collagenase 1 (SIGMA), 0.3 mg/mL DNase I (Boehringer, Mannheim, Germany) and 2 mg/mL hyaluronidase (SIGMA) in PBS (Gibco, Karlsruhe, Germany). The isolated cells were cultured in 25 cm^2 culture flasks (Costar, Bodenheim, Germany) with DMEM containing 10% FCS, penicillin (100 IE/mL), streptomycin (100 μg/mL), ciprofloxacin (8 μg/mL), gentamycin (50 μg/mL) and amphotericin B (1 μg/mL). Non-adherent cells were removed by subsequent changes of medium. The remaining cells were used between passage 3 and 8.

Incubation of CLPF with TGF-β1

CLPF were incubated for two days or six days with 0, 0.1, 1, 10 and 50 ng/mL of TGF-β1 (Biozol, Germany) in serum-free medium. The culture medium of 6 d treated cells was changed after 3 d and new TGF-β1 was added for a further three days. Subsequently, cells were used for investigation of α-SMA production, cell

migration, quantitative mRNA analysis, FN, and FAK production.

Wounding assay: Induction of CLPF migration with platelet derived growth factor-AB

CLPF were incubated for 6 d with or without 10 ng/mL of TGF-β1 (Biozol, Germany) in serum-free medium. The medium was changed after three days and new TGF-β1 was added for a further three days. The cells cultured in 5 cm dishes were wounded with a comb, washed and incubated for a further 4 h with different concentrations of platelet derived growth factor (PDGF)-AB (R&D Systems, Minneapolis, MN, USA; 0, 5, 10, and 20 ng/mL). Cells were harvested and used for quantitative analysis of FN and FN splicing form mRNA and for Western blot analysis of FN and FN-splicing form protein as well as levels of total and phosphorylated FAK.

Immunocytochemical staining

For immunocytochemical staining of α-SMA, 20 000 cells were seeded onto LabTek® Chamber slides (Nunc, Wiesbaden, Germany) and fixed with ice-cold acetone. Immunocytochemical stainings were performed according to the manufacturer's APAAP protocol (Dako, Hamburg, Germany). The anti α-SMA antibody (1:25, clone 1A4, Dako) was used to investigate the differentiation of CLPF into myofibroblasts. IgG2a (MOPC-141, Sigma) was used as an isotype control.

Western blotting

Western blot analysis was performed as described earlier[17,29]. Protein detection was carried out with a specific antibody to α-SMA (clone 1A4, Dako, Hamburg, Germany) and incubation with peroxidase-conjugated secondary antibody. Protein bands were visualized using a commercial chemoluminescence detection kit (ECL Plus; Amersham) according to the manufacturer's protocol. Loading was checked by Ponceau S staining.

For FN and FAK protein immunoblots specific antibodies to FN (1:1000; 35041A, Pharmingen), FN ED-A (1:400; clone IST-9, Abcam), FN ED-B (1:200; PhiloMab-2, Philogen), FAK (1:666; Chemicon), phosphoFAK (1:1000; Biosource) and β-actin (1:10 000; Chemicon) were used. After washing of the membranes horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA) were added and incubated with chemiluminescent substrate (ECL Plus; Amersham Biosciences, Arlington Heights, IL) for 5 min before a film (Amersham Biosciences) was exposed.

After exposure, the antibody was stripped from the membrane with Re-Blot Plus-Strong (Chemicon, Hofheim, Germany) for 15 min and the blot was blocked again with 5% milk in 0.1% Tween 20-TBS before using it for the next antibody.

Migration assays

Migration assays were performed in a modified 48-well Boyden chamber as described earlier[28]: A polycarbonate filter (8 μm pore size, polyvinylpyrrolidone-free, Gerbu Biotechnik, Gaiberg, Germany) divided the chamber into an upper and a lower compartment. Each test substance was placed in the wells of the lower compartment in replicates of three. Conditioned media with or without PDGF-AB, IGF-I (R&D Systems), EGF (Biosource, Germany) or TGF-β1 (Biozol) were tested. DMEM high glucose medium with or without 1% bovine serum albumin (BSA, SIGMA) served as negative controls. A total of 20 000 CLPF/well in DMEM high glucose medium with 1% BSA were seeded into the wells of the upper compartment of the Boyden chamber. The Boyden chamber was incubated at 37℃ in 10% CO_2 atmosphere for 6 h. The filter was removed from the chamber and the non-migrated cells on the upper side of the filter were scraped off with a rubber policeman. The migrated cells on the lower side of the filter were fixed and stained with Hemacolor staining kit (Merck, Darmstadt, Germany) and counted in 4 microscopic high power fields of view (hpf) at a 400-fold magnification. Unless otherwise indicated, each experiment was repeated at least three times (n = 3).

Conditioned media

The culture medium was removed from a confluent CLPF monolayer. The cells were washed twice with PBS and subsequently cultured with DMEM lacking FCS for 24 h. The conditioned medium was centrifuged to remove all cell debris and stored at -20℃ for no more than 3 mo.

RNA isolation and cDNA synthesis

For the isolation of total RNA stimulated and unstimulated primary human CLPF cultures were washed twice with PBS. CLPF were scraped off, centrifuged for 5 min, and resuspended with lysis buffer from the RNeasy® kit (Qiagen, Hilden, Germany). Total RNA was prepared from these CLPF according to the manufacturer's protocol and stored at -80℃. The isolated RNA was reverse transcribed using the Promega Reverse Transcription System (Promega, Madison, WI, USA).

Quantitative mRNA analysis by real-time PCR

Amounts of FN, ED-A and ED-B mRNA were quantified by real-time PCR as previously described[17].

Released reporter dye fluorescence during 40 cycles of amplification was monitored using Sequence Detector software (SDS version 2.0, PE Applied Biosystems). Reporter dye fluorescence versus PCR cycles was plotted. A threshold was set in the exponential phase of the fluorescence curves. The threshold cycle numbers (Ct) were calculated.

Ct values of GAPDH were subtracted from those of FN isoforms: dCt = Ct (FN isoforms) - Ct (GAPDH). The mean value of dCt values was calculated. The values of cDNA from stimulated CLPF were subtracted from those of untreated cDNA of the same CLPF: ddCt = dCt (stimulated) - dCt (unstimulated). The relative start

amount of cDNA was calculated in consideration of the exponential amplification: $x = 2^{-ddCt}$.

Statistical analysis

All data are given as mean ± SE. The Student's *t*-test was used for analysis of parametric data, while the Mann-Whitney Rank Sum Test was used for evaluation of non-parametric data. Real time PCR data were pictured as boxplots. $P < 0.05$ were considered to be statistically significant.

RESULTS

Effect of TGF-β1 on α-SMA production of CLPF

To investigate the effect of TGF-β1 on α-SMA production of CLPF, CLPF were incubated with different concentrations of TGF-β1 in serum-free medium. Subsequently, APAAP-staining of α-SMA or its isotype control IgG2aκ was performed. Since there was no remarkable change in α-SMA production after treatment for one day or two days with 0.1, 1, and 10 ng/mL TGF-β1 (data not shown), we compared α-SMA production of CLPF incubated for two days (Figure 1A) or six days (Figure 1B) with 0, 10 and 50 ng/mL TGF-β1. A time- and dose-dependent increase of α-SMA was observed: short-term treatment with 50 ng/mL TGF-β1 resulted in enhanced α-SMA production, however, after long-term incubation α-SMA was detected in almost all cells.

In order to confirm these results, Western blot analysis was performed (Figure 2A). CLPF treated for two days or six days with 10 ng/mL or 50 ng/mL TGF-β1 were lysed and levels of α-SMA were detected. In each lane 30 μg/mL cell lysate protein were loaded and the loading was checked by Ponceau S staining (Figure 2B). Western blot data confirmed the induction of α-SMA production by long-term incubation of CLPF with TGF-β1 in contrast to short-term incubation, where no α-SMA was detected.

Effect of TGF-β1 on the migration of CLPF

Since TGF-β1 potently stimulated the production of α-SMA in CLPF and as a consequence their differentiation into myofibroblasts, we investigated the effect of long term TGF-β1 incubation on the migration of CLPF. Because 10 ng/mL TGF-β1 induced similar α-SMA levels as 50 ng/mL TGF-β1 (Figure 2), we treated CLPF for six days with 10 ng/mL TGF-β1 and performed migration assays in the presence of PDGF-AB, IGF- I , EGF and TGF-β1 diluted in 24 h CLPF-conditioned medium in a modified Boyden chamber (Figure 3). The Boyden chamber was incubated at 37℃ in 10% CO_2 atmosphere for 6 h. We reported earlier that growth factors like PDGF-AB, IGF- I , EGF and TGF-β1 diluted in conditioned media enhance CLPF migration[28]. However, pre-incubation of CLPF with TGF-β1 for six days significantly decreased CLPF migration. This decreased migratory potential of myofibroblast-like CLPF could not be re-enhanced by addition of growth factors (Figure 3).

Migration assays with different dilutions of PDGF-AB in conditioned medium showed that short-term incubation (6 h) in the modified Boyden chamber enhanced migration of TGF-β1-untreated CLPF in a dose-dependent manner (Figure 3A). Addition of 5 ng/mL PDGF-AB to CLPF conditioned medium increased migration up to 60% (64 ± 5 cells/hpf) when compared to conditioned medium without additional PDGF-AB (control, 44 ± 4 cells/hpf). Addition of 20 ng/mL PDGF-AB to CLPF conditioned medium induced a more than two-fold migration rate (121 ± 19 cells/hpf) when compared to control. Pre-incubation of CLPF for six days with 10 ng/mL TGF-β1 reduced migration to 37% (16 ± 2 cells/hpf) when compared to untreated CLPF (44 ± 4 cells/hpf) and could not be re-enhanced by PDGF-AB ($P < 0.0001$; Figure 3A).

Similar results were obtained in migration assays using different concentrations of IGF- I , EGF or TGF-β1 in conditioned medium (Figure 3B-D). Addition of the growth factors to the conditioned medium enhanced the migration of TGF-β1-untreated CLPF, although the effect was not as strong as with PDGF-AB. Again, pre-incubation of CLPF for six days with TGF-β1 led to a reduced migratory potential of the cells and could not be re-enhanced by the growth factors IGF- I (Figure 3B: 100 ng/mL, $P < 0.005$; 200 ng/mL, $P < 0.05$; 300 ng/mL, $P < 0.0001$), EGF (Figure 3C: 10 ng/mL, $P < 0.05$) or TGF-β1 (Figure 3D: 10 pg/mL, $P < 0.005$; 20 pg/mL, $P < 0.0001$; 30 pg/mL, $P < 0.05$).

Effect of TGF-β1 on the production of FN and FN isoforms

It is known that TGF-β1 promotes the insertion of the ED-A domain in FN[30,31] and we have recently shown that changes in the migratory potential of CLPF were correlated with changes in FN isoform production[17]. For this reason, the effect of TGF-β1 on splicing of FN mRNA in CLPF was assessed. CLPF were incubated with different TGF-β1 concentrations for two days or six days and the amounts of FN, FN ED-A-, and FN ED-B were determined. Therefore the cells were treated with 10 ng/mL and 50 ng/mL TGF-β1 and untreated cells were used as controls. For the comparison of mRNA expression quantified by real time PCR untreated controls were set as 1. The results of 7 independent experiments were evaluated statistically (Figure 4).

After two days incubation with 10 ng/mL TGF-β1 total FN mRNA expression was increased 20-fold and 25-fold with 50 ng/mL (both $P < 0.005$, paired *t*-test). When CLPF were incubated for six days, FN mRNA was increased 6-fold with 10 ng/mL TGF-β1 and 10-fold with 50 ng/mL TGF-β1 compared to control (Figure 4A).

mRNA amounts of FN ED-A and FN ED-B were increased 20-fold ($P < 0.05$) and 13-fold ($P < 0.005$), respectively, with 10 ng/mL TGF-β1 after two days, while after six days 5-fold (FN ED-A, $P < 0.05$) or 3-fold (FN ED-B, $P < 0.05$) increased mRNA levels compared to controls were found (Figure 4B and C). Treatment with 50 ng/mL TGF-β1 for 2 d caused a 23-fold ($P < 0.01$) increase of FN ED-A mRNA which

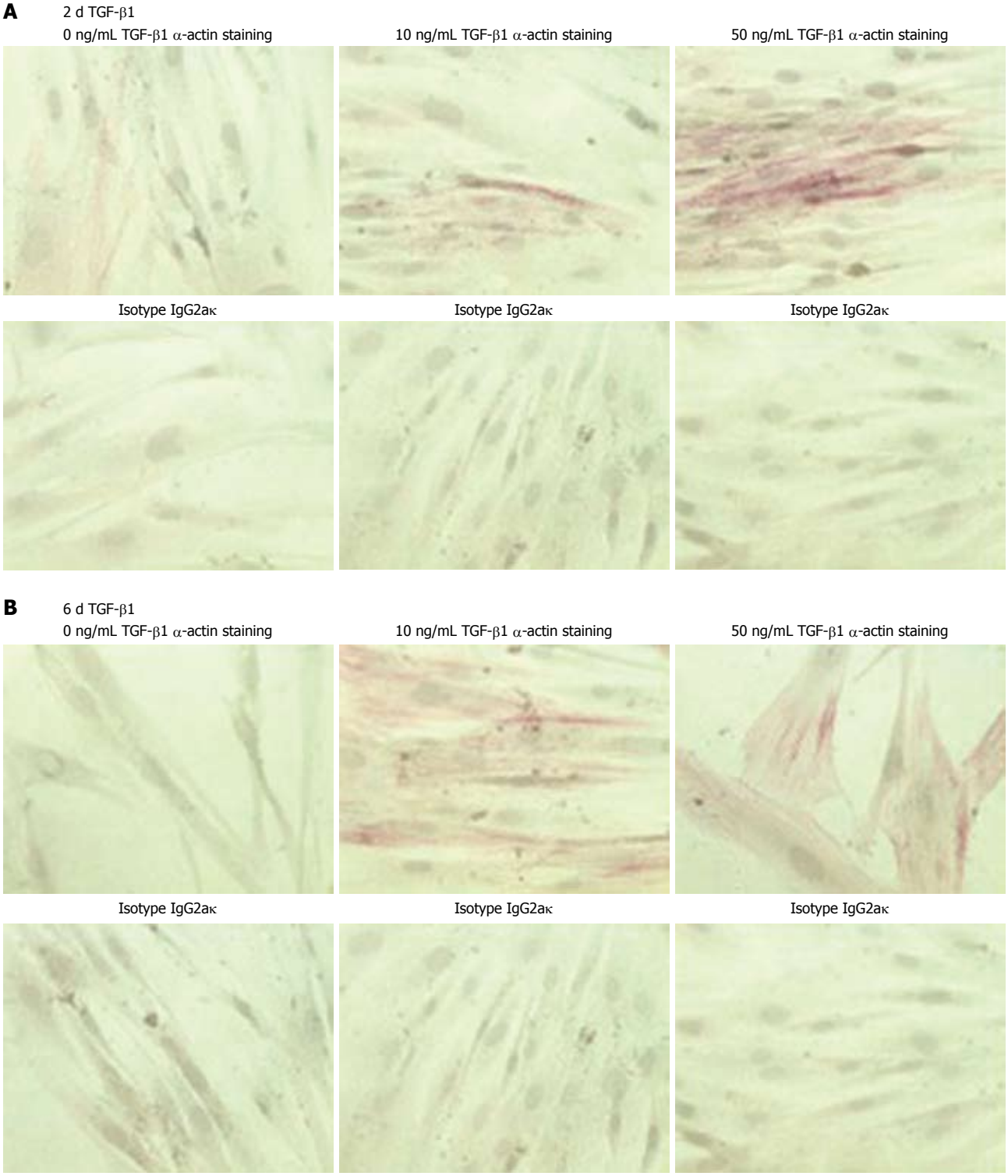

Figure 1 Effect of TGF-β1 on α-SMA production in CLPF. CLPF were incubated for 2 d (A) or 6 d (B) with 0, 10 and 50 ng/mL TGF-β1. Subsequently, APAAP-staining of α-SMA (α-actin) or its isotype control IgG2a was performed. Incubation of CLPF for 6 d with 10 ng/mL or 50 ng/mL TGF-β1 showed the greatest effect on α-SMA, since almost all cells showed a red stainining for α-SMA.

was reduced to 6-fold after a further four days. mRNA amounts of FN ED-B was 15-fold ($P < 0.01$) after two days and reduced to 4-fold ($P < 0.05$) after six days (Figure 4B and C).

Together these data indicate that TGF-β1 causes a strong increase in mRNA expression of FN and migration inducing FN isoforms. However, this effect was not maintained but was clearly reduced after six days. Values obtained after six days of incubation were not statistically different to unstimulated controls despite the presence of TGF-β1. On average, FN mRNA amounts were 70% lower after pre-incubation with 10 ng/mL TGF-β1 for six days than after pre-incubation for two days ($P < 0.05$), while FN ED-A and FN ED-B ($P < 0.05$) mRNA levels were reduced by 80%. Similar data were obtained for incubation with 50 ng/mL

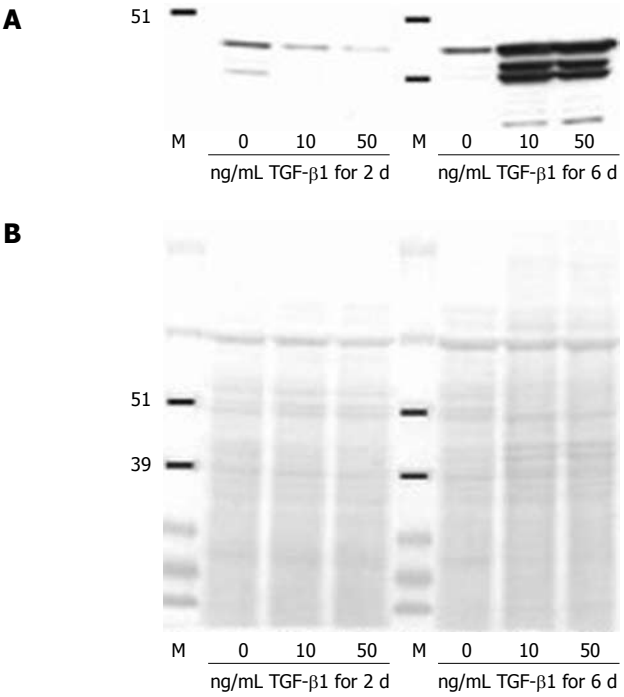

Figure 2 Effect of TGF-β1 on α-SMA protein production. Control CLPF were incubated for 2 d or 6 d with 0, 10, and 50 ng/mL TGF-β1 in serum-free medium. Subsequently the cells were washed and lysed. Amounts of α-SMA were analyzed by Western blotting (A). Equal loading was confirmed by Ponceau S staining (B). Incubation of CLPF for 6 d with 10 ng/mL or 50 ng/mL TGF-β1 markedly enhanced α-SMA production.

TGF-β1 for six days *versus* stimulation for two days (total FN 55% lower ($P < 0.05$) after six days compared to two days, FN ED-A and FN ED-B 75% lower ($P = 0.05$).

In addition, TGF-β1-mediated induction of FN and FN splice variant production was confirmed by Western blot analyses. A time- and dose-dependent increase of total FN, FN ED-A and FN ED-B was detected after TGF-β1 treatment (Figure 5). In contrast to the findings for mRNA expression after six days TGF treatment higher levels of FN, ED-A and ED-B protein compared to two days were observed. In the absence of TGF-β1, levels of FN and FN isoforms were low and did not differ between two- and six- day cultures. A concentration of 0.1 ng/mL TGF-β1 did not show a significant effect while 1 ng/mL TGF-β1 induced an increase in FN and FN ED-A production after two days and six days compared to untreated cells. After two days FN ED-B protein was only found after incubation with TGF-β1 concentrations of 10 ng/mL and 50 ng/mL, while this isoform could be detected after six days with 1, 10, and 50 ng/mL TGF-β1. The most prominent effect on FN, FN ED-A, and FN ED-B production was observed after six days incubation with 50 ng/mL TGF-β1.

Effects of TGF-β1 and PDGF-AB on FN and FN isoforms in a wounding model

Because of the observed reduced migratory potential of CLPF incubated long-term with TGF-β1 but increased protein levels of migration inducing factor FN and FN splice variants, we analyzed migrating cells in a wounding assay in more detail. CLPF were pre-incubated with and without 10 ng/mL TGF-β1 in serum free medium (six days). The CLPF monolayer was wounded with a comb, migration was induced by incubation with different concentrations of PDGF-AB (0, 5, 10, 20 ng/mL) in conditioned medium for 4 further hours and mRNA amounts of FN isoforms were assessed (Figure 6).

While CLPF without TGF-β1 pre-treatment displayed a slight PDGF-dependent increase in the expression of FN, FN ED-A, and a slight decrease in FN ED-B mRNA in this wounding assay, TGF-β1 pre-treated cells displayed a stronger enhancement of FN, FN ED-A, and FN ED-B mRNA-expression.

After stimulation with 5, 10, and 20 ng/mL PDGF-AB in conditioned medium FN mRNA-expression was 4.5- (Paired *t*-test, $P < 0.05$), 4- ($P < 0.01$), and 4.5-fold ($P < 0.05$) increased in TGF-β1 pre-treated and 2-, 3.5-, and 1.5-fold enhanced in TGF-β1 untreated CLPF as compared to the control, respectively (Figure 6A). PDGF-AB stimulation of pre-incubated CLPF led to 3- (5 ng/mL PDGF, $P < 0.005$), 2.5- (10 ng/mL PDGF, $P < 0.01$), and 4-fold increases (20 ng/mL PDGF, $P < 0.05$) in FN ED-A mRNA expression, whereas TGF-β1 unstimulated cells showed a 2- ($P < 0.05$), 3.5-, and 1.5-fold enhancement, respectively (Figure 6B). PDGF caused a 3- (5 ng/mL, $P < 0.01$), 2.5- (10 ng/mL, $P < 0.01$), and 3-fold increase (20 ng/mL, $P < 0.01$) in FN ED-B mRNA in TGF-β1-treated CLPF and a 1.5-, 2.5- (5 and 10 ng/ mL, $P < 0.01$), and 1.5-fold (20 ng/mL) enhancement in TGF-β1 untreated cells (Figure 6C).

In summary, pre-treatment of CLPF with TGF-β1 (six days) and subsequent treatment with PDGF-AB (4 h) after wounding causes a much stronger induction of FN mRNA expression than without TGF-β1 pre-treatment.

After TGF-β1-incubation, wounding, and incubation without PDGF-AB mRNA levels of total FN, FN ED-A, and FN ED-B increased significantly by 4-fold when compared to TGF-β1- and PDGF-AB-untreated controls ($P < 0.05$ for FN and FN ED-A; FN ED-B $P < 0.005$). With 5 ng/mL PDGF-AB mRNA amounts of FN splice forms doubled by TGF-β1-stimulation [FN ED-A ($P < 0.05$)]. After TGF-β1 pre-stimulation expression of FN and FN ED-B rose by 20% compared to untreated cells, while FN ED-A expression was even decreased by 30%. With 10 ng/mL TGF-β1 and 20 ng/mL PDGF-AB, after cell wounding total FN expression showed a 3-fold ($P < 0.05$), FN ED-A expression a 2.5-fold ($P = 0.06$), and FN ED-B expression a 2-fold ($P < 0.005$) increase.

Western blot analyses of protein production confirmed these findings on mRNA expression (Figure 7). Protein amounts of FN, FN ED-A, and FN ED-B were strongly increased by TGF-β1 incubation. In comparison to TGF-β1 untreated controls the levels of FNs were enhanced after wounding and further enhanced by addition of PDGF-AB in conditioned medium. No significant differences were observed between protein amounts in TGF-β1 pre- and not incubated CLPF by PDGF-treatment, respectively. FN

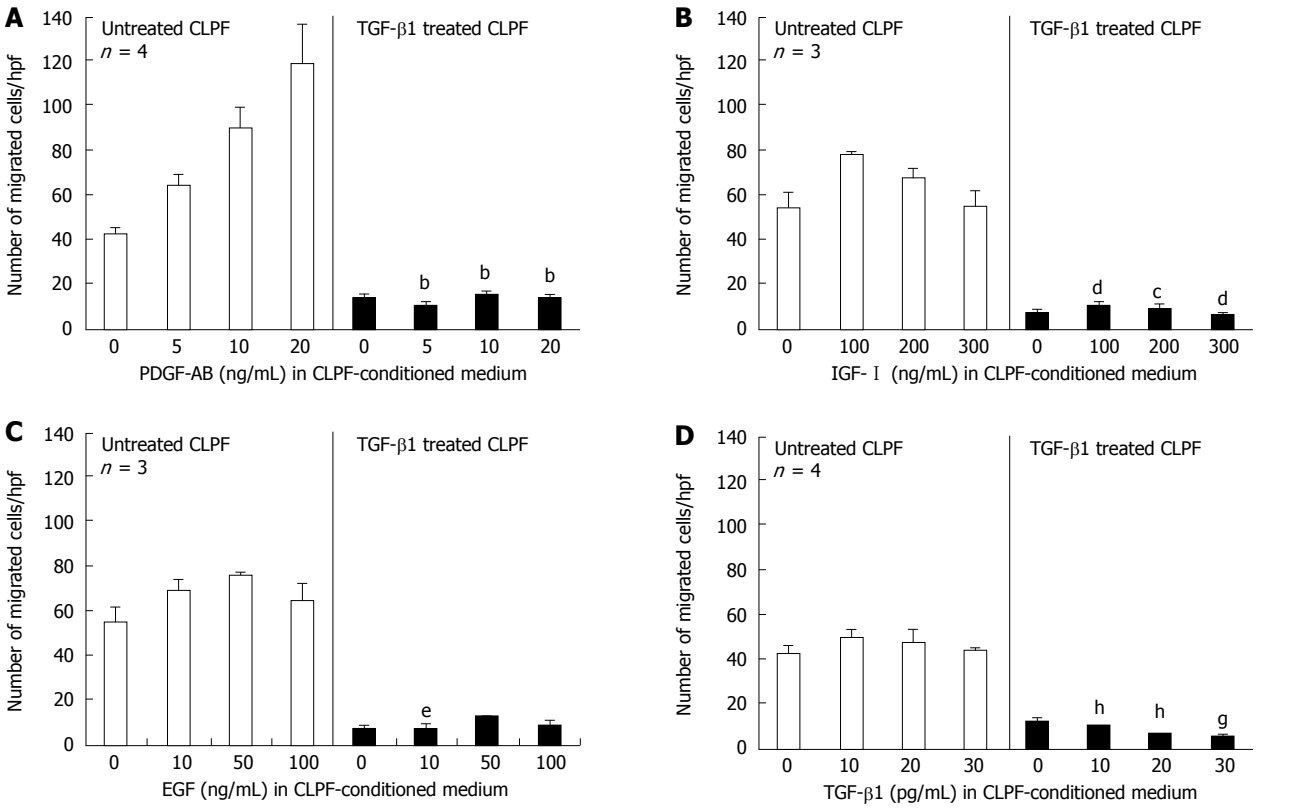

Figure 3 Effect of TGF-β1 on growth factor-induced migration of CLPF. Control-CLPF were treated for 6 d with 10 ng/mL TGF-β1 and subsequently migration assays in the presence of PDGF-AB, IGF-Ⅰ, EGF and TGF-β1 diluted in 24 h CLPF-conditioned medium were performed in the modified Boyden chamber. TGFβ1 treatment of CLPF significantly decreased the migratory potential, which could not be re-enhanced by cell migration-inducing growth factors. Statistics: The Mann-Whitney Rank Sum Test was used for the evaluation of the non-parametric data. A: $^{b}P < 0.0001$ *vs* unstimulated CLPF; B: $^{c}P < 0.05$, $^{d}P < 0.005$ *vs* unstimulated CLPF; C: $^{e}P < 0.05$ *vs* unstimulated CLPF; D: $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* unstimulated CLPF.

ED-B protein levels of CLPF without pre-treatment were barely detectable.

Effects of TGF-β1 on FAK phopshorylation in a wounding model

We have demonstrated in previous studies that enhanced cell migration of CLPF was associated with higher FAK phosphorylation and total FAK protein production[29]. Therefore, we determined TGF-β1-modulated FAK production and phosphorylation by Western blot (Figure 8) in CLPF that were induced to migrate by wounding and stimulation with PDGF-AB in conditioned medium. Pre-incubation of CLPF with 10 ng/mL TGF-β1 for six days decreased FAK phosphorylation as compared to untreated controls. In addition, levels of FAK protein were reduced by TGF-β1 stimulation. CLPF that were not pre-treated with TGF-β1 contained enhanced levels of FAK with a constant higher FAK phosphorylation that was highest with an addition of 5 ng/mL PDGF-AB when compared to CLPF not treated with TGF-β1.

DISCUSSION

In this study we demonstrate that TGF-β1 potently stimulates the production of α-SMA in CLPF and therefore their differentiation into myofibroblasts as a result of long term contact. This differentiation into myofibroblasts is accompanied by a reduced migratory potential, reduced FAK phosphorylation and increased synthesis of FN as a component of extracellular matrix (ECM).

Myofibroblast differentiation and activation by TGF-β1 is a critical event in tissue repair and the pathogenesis of human fibrotic diseases. Myofibroblasts and TGF-β1 are key elements for the generation of contractile force associated with wound contraction and pathological contractures as in the development of tissue fibrosis. Myofibroblasts are characterized by the presence of α-SMA-containing stress fibers, vinculin-containing fibronexus adhesion complexes, and FN fibrils containing the ED-A splice variant[32]. TGF-β1 promotes contraction of collagen gels by fibroblasts through their differentiation into myofibroblasts[33]. In addition, TGF-β1 induces a dose-dependent increase in the generation of contractile force and a concomitant increase in the production of α-SMA[32].

In the current study much higher TGF-β1 doses were required to induce α-SMA production than in the studies by Simmons *et al*[9] and Vaughan *et al*[32]. This may be a cell-type-specific effect. Simmons et al. used CCD-18Co fibroblasts isolated from the colon of a 2.5 mo old child, while in this study intestinal fibroblasts of adults have been used. Vaughan *et al*[32] used myofibroblasts obtained as explant cultures or by collagenase digestion of palmar aponeurosis from patients with Dupuytren's disease. Therefore, it may be

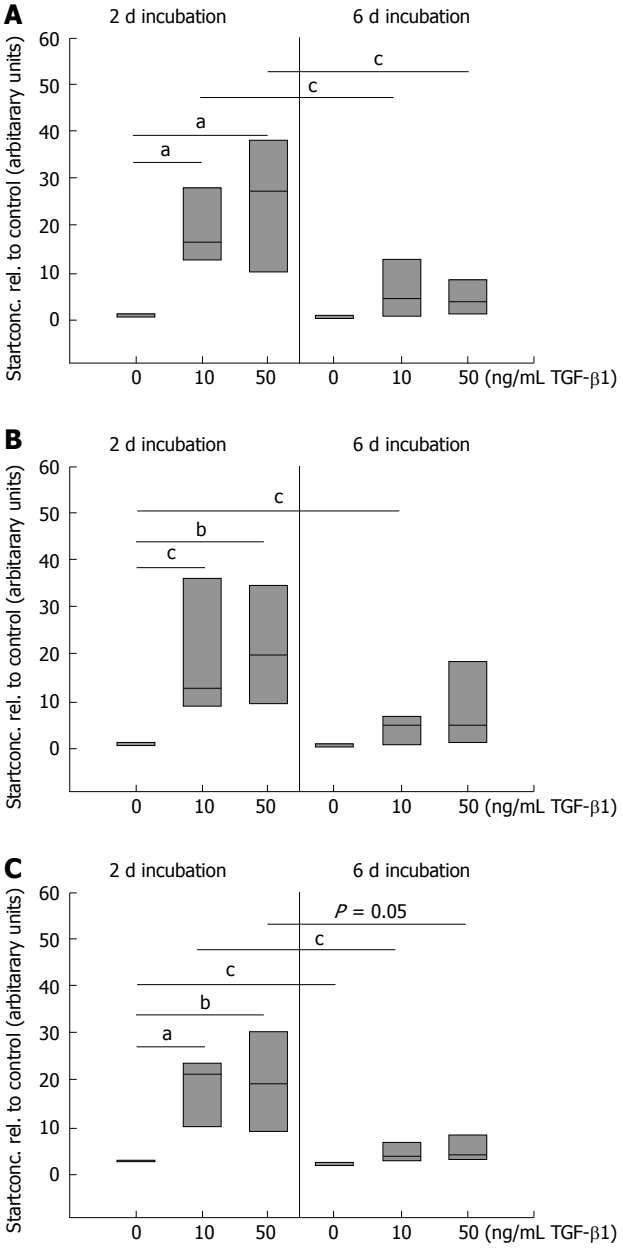

Figure 4 Quantitative mRNA analysis of FN (A) and the splicing forms FN ED-A (B) and FN ED-B (C) by real-time PCR in TGF stimulated CLPF. TGF-β1 stimulated and untreated control-CLPF (n = 7) were lysed and mRNA was isolated. The cDNA start concentration of the untreated control group was set as 1. Compared to the control cells a dose-dependent increase in mRNA expression of FN, FN ED-A, and FN ED-B was determined. TGF-β1-stimulation for 2 d caused a higher increase in FN mRNA expression than after 6 d. Paired t-test: $^{a}P < 0.005$; $^{b}P < 0.01$; $^{c}P < 0.05$.

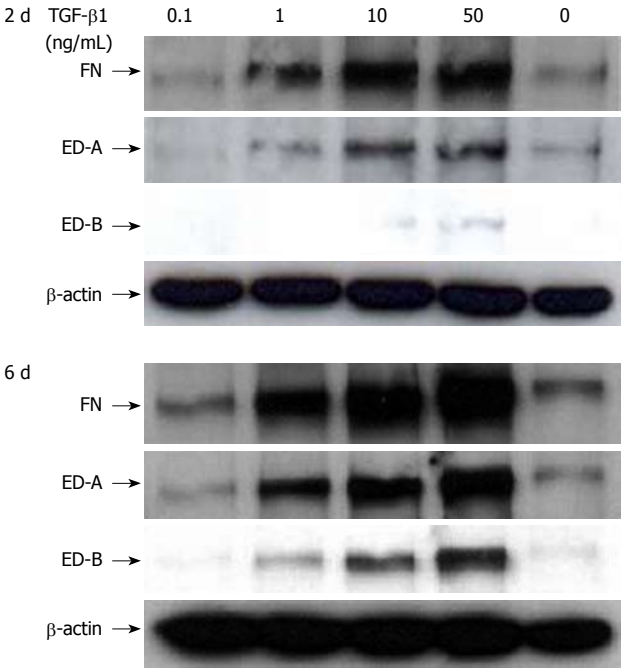

Figure 5 Effect of TGF-β1 on FN, FN ED-A, and FN ED-B protein in CLPF after 2 d and 6 d. Control-CLPF were incubated for 2 d or 6 d with 0, 0.1, 1, 10, and 50 ng/mL TGF-β1 in serum-free medium. Subsequently cells were washed and lysed. Amounts of FN were quantified by Western blotting. Loading was checked by β-actin production. Incubation of CLPF for 6 d with TGF-β1 markedly enhanced the levels of FN, FN ED-A, and FN ED-B.

that fibroblasts obtained from palmar aponeurosis react more sensitively to TGF-β1 than CLPF.

Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling *via* FAK. TGF-β1 induces tyrosine phosphorylation of the autophosphorylation site Tyr-397 of FAK, an effect that is dependent on cell adhesion and is delayed relative to early Smad signaling. Pharmacologic inhibition of FAK or expression of kinase-deficient FAK, mutated by substituting Tyr-397 with Phe, inhibited TGF-β1-induced α-SMA production and stress fiber formation[34]. In the current study we found that long term incubation with TGF-β1 reduced FAK phosphorylation and this was associated with a decreased migratory potential. TGF-β1 untreated CLPF displayed a PDGF-dependent increase of FAK-phosphorylation that correlated with an enhanced migration.

FAK is a nonreceptor protein tyrosine kinase involved not only in adhesion but also in cell migration. FAK-deficient fibroblasts exhibit defects in cell migration and elevated numbers of cell-substratum contact sites[35]. The reduced migratory behavior of CD- and ulcerative colitis (UC)-CLPF is accompanied by a reduction of FAK and FAK phosphorylation[29].

TGF-β1 production is increased in myofibroblasts at sites of fibrosis in experimental enterocolitis and CD[9]. In CD, increased TGF-β1 expression is transmural whereas in UC, the increase is confined to the lamina propria and submucosa. The distribution of TGF-β1 coincides with the distribution of the inflammatory infiltrate as well as an increase in the collagen type Ⅲ: Ⅰ ratio in both CD and UC[36]. Additionally, TGF-β1, -β2, -β3 and their receptors are increased in fibrotic CD mucosal tissue samples[13]. Therefore, it may be assumed that a reduced migratory potential of inflammatory bowel disease (IBD)-CLPF[29] is a result of enhanced α-SMA production and enhanced formation of focal contacts induced by increased levels of TGF-β1 in the tissue. However, we could not find higher α-SMA levels in *ex vivo* cultures of CD- and UC-CLPF compared to cultures isolated from control mucosa (data not shown). Therefore, a differentiation of IBD-CLPF into myofibroblasts was not the reason for the reduced migratory potential of these cells.

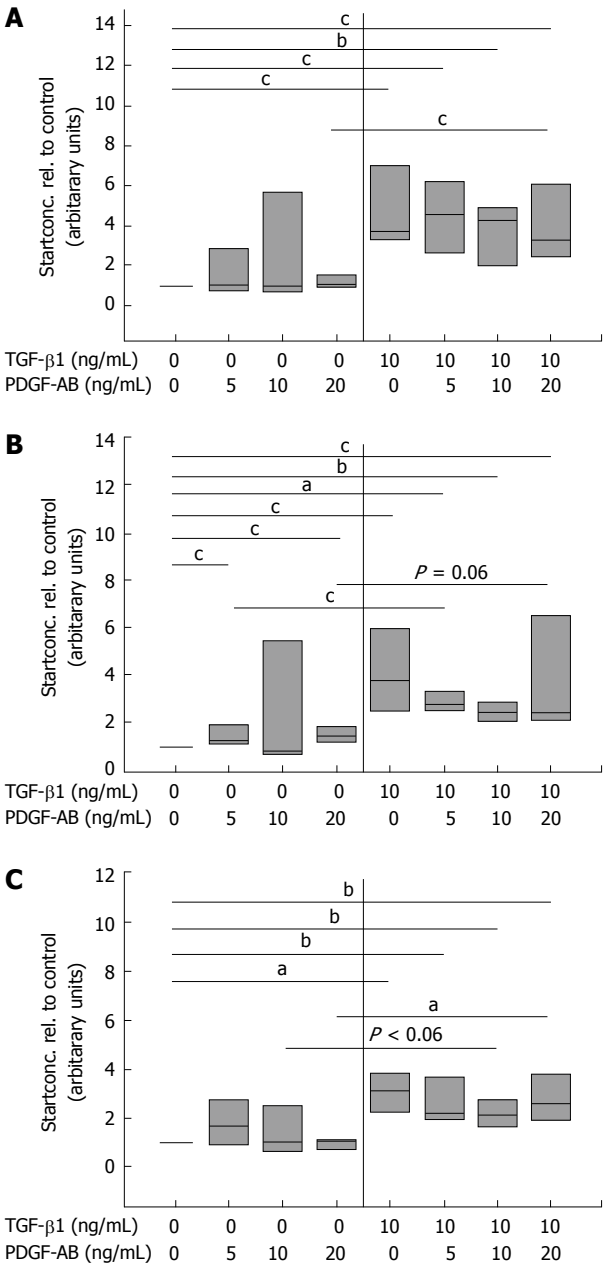

Figure 6 Quantitative mRNA analyses of FN (A) and the splicing forms FN ED-A (B), and FN ED-B (C) by real-time PCR in CLPF treated with TGF-β1. Control CLPF (n = 7) were pre-incubated with and without 10 ng/mL TGF-β1 for 6 d, the monolayer wounded with a comb, and incubated for further 4 h with increasing concentrations of PDGF-AB in conditioned medium. mRNA was isolated and cDNA transcribed. cDNA start concentration of the untreated control (0 ng/mL TGF-β1 and 0 ng/mL PDGF-AB) was set as 1. Paired t-test: $^{a}P < 0.005$; $^{b}P < 0.01$; $^{c}P < 0.05$.

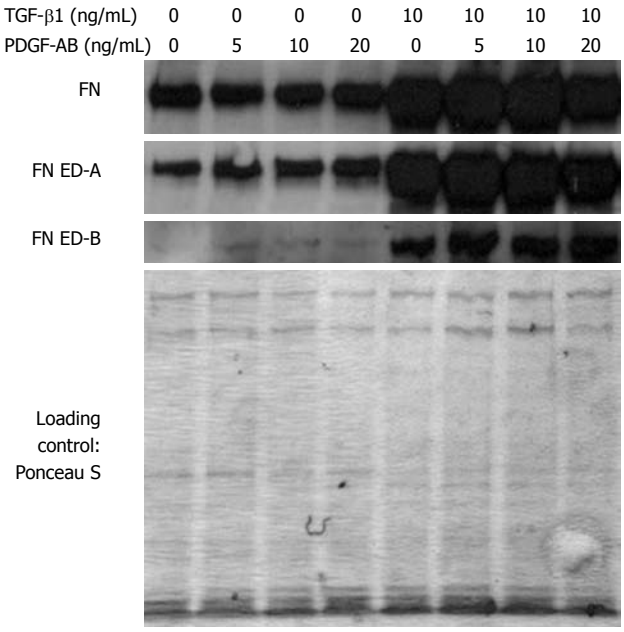

Figure 7 FN, FN ED-A, and FN ED-B protein levels of TGF-β1-pre-stimulated and unstimulated control CLPF in a wounding assay. Isolated proteins were analyzed by Western blotting. Protein levels of FN, FN ED-A, and FN ED-B were increased by TGF-β1-pre-treatment compared to untreated control. Loading was checked by Ponceau S staining.

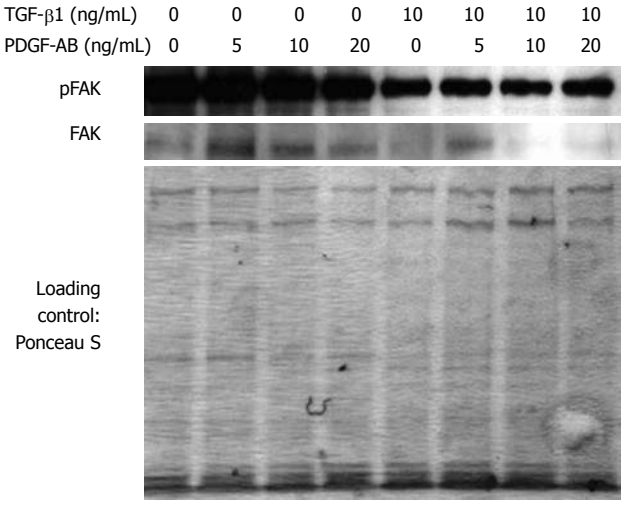

Figure 8 Analysis of FAK phosphorylation and FAK protein production of CLPF after TGF-β1 incubation in a wounding assay. Isolated proteins were analyzed by Western blotting. Phospho-FAK and FAK decreased after 6 d TGF-β1 pretreatment in comparison to untreated controls. Loading was checked by Ponceau S staining.

Contrasting migration-modulating effects of TGF-B are described in the literature. Ellis *et al*[7] report a diverse pattern of motogenic response to the three TGF-B isoforms. Migration of subconfluent fibroblasts into 3D collagen gels was inhibited by all three TGF-B-isoforms, whereas migration of confluent cells was unaffected by TGF-β1 and TGF-β2, but stimulated by TGF-β3[7]. Postlethwaite *et al*[8] reported that TGF-β1 is a potent chemoattractant in the Boyden chamber for human dermal fibroblasts. Inhibitory effects of TGF-β1 on fibroblast migration into 3D collagen gels are in marked contrast to reports indicating that TGF-β1 stimulates the migration of human skin fibroblasts in the modified Boyden chamber[6].

Previously we investigated the effect of TGF-β1 on CLPF migration in the modified Boyden chamber after a 6 h incubation period[28] and found a significant migration-inducing effect of TGF-β1. Here we report that long term incubation for 6 d with TGF-β1 induces a marked reduction of migration in subsequent migration assays. This discrepancy obviously was determined by the time TGF-β1 could act on the target cells.

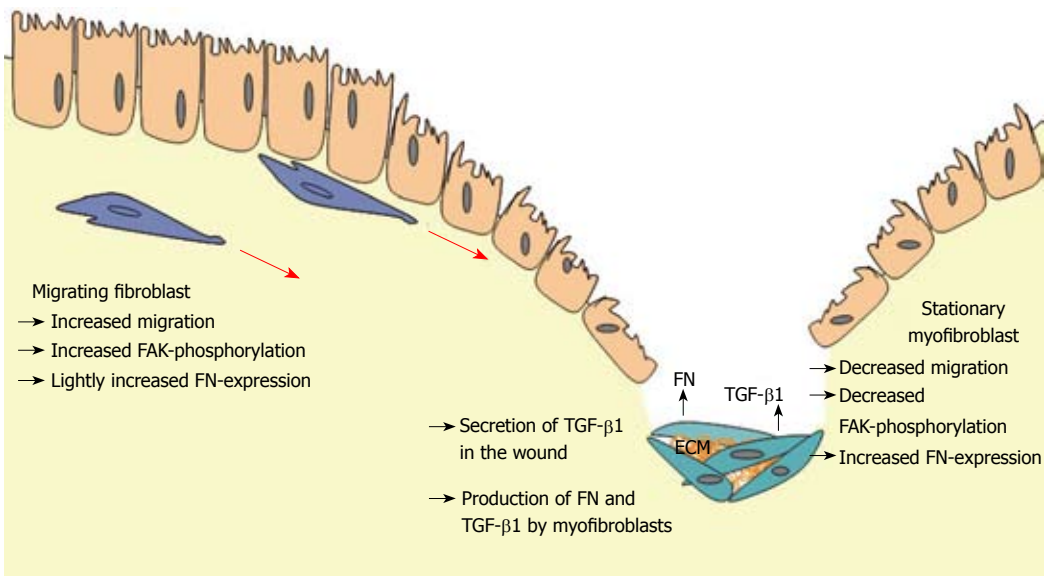

Figure 9 Schematic illustration of the role of TGF-β1, FN, and FAK in the development of fibrosis in CD. After wounding TGF-β1 is produced by different cell types and connective tissue cells. CLPF increase FAK phosphorylation and migrate into the wound along the TGF-β1 gradient. FN that is also produced during migration induction and secreted at the site of injury may lead to an additional enhancement of the migratory gradient. Long term contact with TGF-β1 within the wound allows fibroblasts to differentiate into myofibroblasts. These cells have a reduced migratory potential and support wound healing via wound contraction and production of extracellular matrix deposition like FN. Phosphorylation of FAK is reduced after longer contact with TGF-β1 due to the differentiation into ECM-producing myofibroblasts.

Addition of 10 ng/mL and 50 ng/mL TGF-β1 resulted in a dose-dependent increase of FN, FN ED-A, and FN ED-B expression. After two days incubation, expression of FN and the splice forms significantly increased. Enhanced mRNA expression was also observed after 6 d incubation with TGF-β1 but was not as high as after 2 d. On the other hand, after six days higher protein levels of total FN and FN isoforms were observed in comparison to the unstimulated two day control. Therefore, the reduced migration of CLPF after six days incubation with TGF-β1 is accompanied by enhanced protein levels of FN and FN isoforms. This increased production of FN and FN isoforms might lead to enhanced cell adhesion. Other groups also report that fibroblast attachment is significantly increased after TGF-β1 treatment[36]. Nevertheless, cell adhesion was not addressed in our investigations.

In CLPF treated for six days with TGF-β1, after wounding and PDGF-AB incubation increased protein levels of FN, FN ED-A, and FN ED-B were found when compared to untreated controls. In these cells FAK protein and FAK phosphorylation were reduced. This reduced FAK phosphorylation correlated with the observed reduced migratory potential after long term incubation with TGF-β1.

In conclusion, long term incubation of intestinal fibroblasts with TGF-β1 induced differentiation into myofibroblasts with an enhanced α-SMA production and a reduced migratory potential that was accompanied by decreased FAK production and phosphorylation. In contrast, short term contact of CLPF with TGF-β1 induced a dose-dependent increase of cell migration without induction of α-SMA. Therefore, the following scenario is conceivable: after wounding TGF-β1 is produced by different cell types and connective tissue cells, like CLPF, migrate towards the wound along the TGF-β1 gradient. Long-term contact with TGF-β1 in the wound allows the fibroblasts to differentiate into myofibroblasts. Myofibroblasts support wound healing *via* wound contraction and production of extracellular matrix deposition. In pathological situations the local increase in immigrated cell numbers with reduced dispersing potential and increased deposition of extracellular matrix may finally lead to tissue fibrosis. FN that is produced during the induction of migration and secreted at the site of injury may lead to an additional enhancement of the migratory gradient (Figure 9). The phosphorylation of FAK is reduced after longer contact with TGF due to the differentiation into ECM-producing myofibroblasts.

However, this model remains speculative, since the modulating influence of other factors on fibroblast migration and the complex interactions of fibroblasts with immune cells or epithelial cells certainly play an important role in wound healing, too.

The role of TGF-β and the mechanism of mucosal wound healing or intestinal tissue fibrosis require further investigation to develop therapies for the modulation of the mucosal healing response in IBD.

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COMMENTS

Background

Migration of colonic lamina propria fibroblasts (CLPF) plays an important role during the progression of fibrosis and fistulae in Crohn's disease. Transforming growth factor-β (TGF-β) is involved in the regulation of cell migration, cell differentiation, extracellular matrix deposition, and immune responses. In recent studies, the authors found that CLPF conditioned media induced migration of primary human CLPF and that fibronectin (FN) was mainly responsible for the autocrine induction of CLPF migration. Since the regulation of migration and differentiation of intestinal fibroblasts is an important mechanism during intestinal wound healing and fibrosis, the effect of TGF-β1 on these processes and on FN and FN isoform production was investigated in this study.

Research frontiers

A high number of patients with Crohn's disease have to undergo surgery because of fibrotic disease. The mechanism of stricture formation and the role of TGF-β in this are not well understood.

Innovations and breakthroughs

This study adds to the understanding of the role of TGF-β in intestinal wound healing and stricture formation. It shows that TGF-β can both be beneficial and detrimental depending on the duration of secretion.

Terminology

Colon lamina propria fibroblasts (CLPF): CLPF are mesenchymal cells of connective tissue that are in an activated state and locally agile. After differentiation into myofibroblasts cells are in a less active state, concerned with maintenance. Transforming growth factor (TGF): TGF-β1 is a cytokine that on the one hand stimulates migration of fibroblasts and on the other hand potently stimulates the production of α-smooth muscle actin and stress fiber formation in fibroblasts and therefore their differentiation into myofibroblasts. Fibronectin (FN): FN is an extracellular matrix protein that is mainly responsible for the autocrine induction of CLPF migration and is essentially required for the induction of CLPF migration. Focal adhesion kinase (FAK): FAK is a nonreceptor protein tyrosine kinase involved not only in adhesion but also in cell migration. Enhanced migration of CLPF is associated with higher FAK phosphorylation and total FAK protein production. Myofibroblast differentiation by TGF-β1 is dependent on cell adhesion and integrin signaling *via* FAK.

Peer review

The authors investigated the effects of transforming growth factor-β (TGF-β) on the differentiation of colonic lamina propria fibroblasts (CLPF) into myofibroblasts *in vitro*. They found that long term incubation of CLPF with TGF-β1 induced differentiation into myofibroblasts with enhanced α-SMA, reduced migratory potential and FAK-phosphorylation, as well as increased FN production. In contrast, short term contact (6 h) of fibroblasts with TGF-β1 induced a dose-dependent increase of cell migration and FAK-phosphorylation without induction of α-SMA production. The study is well designed, the methods they use are sound and their results are reliable.

REFERENCES

1 **Kay EP**, Lee MS, Seong GJ, Lee YG. TGF-beta s stimulate cell proliferation via an autocrine production of FGF-2 in corneal stromal fibroblasts. *Curr Eye Res* 1998; **17**: 286-293

2 **Strutz F**, Zeisberg M, Renziehausen A, Raschke B, Becker V, van Kooten C, Müller G. TGF-beta 1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2). *Kidney Int* 2001; **59**: 579-592

3 **Thannickal VJ**, Aldweib KD, Rajan T, Fanburg BL. Upregulated expression of fibroblast growth factor (FGF) receptors by transforming growth factor-beta1 (TGF-beta1) mediates enhanced mitogenic responses to FGFs in cultured human lung fibroblasts. *Biochem Biophys Res Commun* 1998; **251**: 437-441

4 **Adelmann-Grill BC**, Wach F, Cully Z, Hein R, Krieg T. Chemotactic migration of normal dermal fibroblasts towards epidermal growth factor and its modulation by platelet-derived growth factor and transforming growth factor-beta. *Eur J Cell Biol* 1990; **51**: 322-326

5 **Andresen JL**, Ehlers N. Chemotaxis of human keratocytes is increased by platelet-derived growth factor-BB, epidermal growth factor, transforming growth factor-alpha, acidic fibroblast growth factor, insulin-like growth factor-I, and transforming growth factor-beta. *Curr Eye Res* 1998; **17**: 79-87

6 **Ellis I**, Grey AM, Schor AM, Schor SL. Antagonistic effects of TGF-beta 1 and MSF on fibroblast migration and hyaluronic acid synthesis. Possible implications for dermal wound healing. *J Cell Sci* 1992; **102**: 447-456

7 **Ellis IR**, Schor SL. Differential motogenic and biosynthetic response of fetal and adult skin fibroblasts to TGF-beta isoforms. *Cytokine* 1998; **10**: 281-289

8 **Postlethwaite AE**, Keski-Oja J, Moses HL, Kang AH. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J Exp Med* 1987; **165**: 251-256

9 **Simmons JG**, Pucilowska JB, Keku TO, Lund PK. IGF-I and TGF-beta1 have distinct effects on phenotype and proliferation of intestinal fibroblasts. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G809-G818

10 **Shimao Y**, Nabeshima K, Inoue T, Koono M. Role of fibroblasts in HGF/SF-induced cohort migration of human colorectal carcinoma cells: fibroblasts stimulate migration associated with increased fibronectin production via upregulated TGF-beta1. *Int J Cancer* 1999; **82**: 449-458

11 **Eickelberg O**, Köhler E, Reichenberger F, Bertschin S, Woodtli T, Erne P, Perruchoud AP, Roth M. Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am J Physiol* 1999; **276**: L814-L824

12 **Wells RG**. Fibrogenesis. V. TGF-beta signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G845-G850

13 **McKaig BC**, Hughes K, Tighe PJ, Mahida YR. Differential expression of TGF-beta isoforms by normal and inflammatory bowel disease intestinal myofibroblasts. *Am J Physiol Cell Physiol* 2002; **282**: C172-C182

14 **Piek E**, Ju WJ, Heyer J, Escalante-Alcalde D, Stewart CL, Weinstein M, Deng C, Kucherlapati R, Bottinger EP, Roberts AB. Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 2001; **276**: 19945-19953

15 **Badid C**, Mounier N, Costa AM, Desmoulière A. Role of myofibroblasts during normal tissue repair and excessive scarring: interest of their assessment in nephropathies. *Histol Histopathol* 2000; **15**: 269-280

16 **Leeb SN**, Vogl D, Grossmann J, Falk W, Schölmerich J, Rogler G, Gelbmann CM. Autocrine fibronectin-induced migration of human colonic fibroblasts. *Am J Gastroenterol* 2004; **99**: 335-340

17 **Brenmoehl J**, Lang M, Hausmann M, Leeb SN, Falk W, Schölmerich J, Göke M, Rogler G. Evidence for a differential expression of fibronectin splice forms ED-A and ED-B in Crohn's disease (CD) mucosa. *Int J Colorectal Dis* 2007; **22**: 611-623

18 **Hynes R**. Molecular biology of fibronectin. *Annu Rev Cell Biol* 1985; **1**: 67-90

19 **Owens RJ**, Kornblihtt AR, Baralle FE. Fibronectin, the generation of multiple polypeptides from a single gene. *Oxf Surv Eukaryot Genes* 1986; **3**: 141-160

20 **Schwarzbauer JE**. Fibronectin: from gene to protein. *Curr Opin Cell Biol* 1991; **3**: 786-791

21 **Vartio T**, Laitinen L, Närvänen O, Cutolo M, Thornell LE, Zardi L, Virtanen I. Differential expression of the ED sequence-containing form of cellular fibronectin in embryonic and adult human tissues. *J Cell Sci* 1987; **88**: 419-430

22 **Zardi L**, Carnemolla B, Siri A, Petersen TE, Paolella G, Sebastio G, Baralle FE. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J* 1987; **6**: 2337-2342

23 **Kornblihtt AR**, Umezawa K, Vibe-Pedersen K, Baralle FE. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J* 1985; **4**: 1755-1759

24 **MacLeod JN**, Burton-Wurster N, Gu DN, Lust G. Fibronectin mRNA splice variant in articular cartilage lacks bases encoding the V, III-15, and I-10 protein segments. *J Biol Chem* 1996; **271**: 18954-18960

25 **Schwarzbauer JE**, Tamkun JW, Lemischka IR, Hynes RO. Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell* 1983; **35**: 421-431

26 **Ffrench-Constant C**, Van de Water L, Dvorak HF, Hynes RO. Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J Cell Biol* 1989; **109**: 903-914

27 **Nedelec B**, Ghahary A, Scott PG, Tredget EE. Control of wound contraction. Basic and clinical features. *Hand Clin* 2000; **16**: 289-302

28 **Leeb SN**, Vogl D, Falk W, Schölmerich J, Rogler G, Gelbmann CM. Regulation of migration of human colonic myofibroblasts. *Growth Factors* 2002; **20**: 81-91

29 **Leeb SN**, Vogl D, Gunckel M, Kiessling S, Falk W, Göke M, Schölmerich J, Gelbmann CM, Rogler G. Reduced migration of fibroblasts in inflammatory bowel disease: role of inflammatory mediators and focal adhesion kinase. *Gastroenterology* 2003; **125**: 1341-1354

30 **Balza E**, Borsi L, Allemanni G, Zardi L. Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS Lett* 1988; **228**: 42-44

31 **Inoue T**, Nabeshima K, Shimao Y, Koono M. Hepatocyte growth Factor/Scatter factor (HGF/SF) is a regulator of fibronectin splicing in MDCK cells: comparison between the effects of HGF/SF and TGF-beta1 on fibronectin splicing at the EDA region. *Biochem Biophys Res Commun* 1999; **260**: 225-231

32 **Vaughan MB**, Howard EW, Tomasek JJ. Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res* 2000; **257**: 180-189

33 **Lijnen P**, Petrov V, Rumilla K, Fagard R. Transforming growth factor-beta 1 promotes contraction of collagen gel by cardiac fibroblasts through their differentiation into myofibroblasts. *Methods Find Exp Clin Pharmacol* 2003; **25**: 79-86

34 **Thannickal VJ**, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem* 2003; **278**: 12384-12389

35 **Sieg DJ**, Hauck CR, Schlaepfer DD. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci* 1999; **112**: 2677-2691

36 **Lawrance IC**, Maxwell L, Doe W. Inflammation location, but not type, determines the increase in TGF-beta1 and IGF-1 expression and collagen deposition in IBD intestine. *Inflamm Bowel Dis* 2001; **7**: 16-26

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ORIGINAL ARTICLES

Down-regulation of extracellular signal-regulated kinase 1/2 activity in P-glycoprotein-mediated multidrug resistant hepatocellular carcinoma cells

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Abstract

AIM: To study the expression and phosphorylation of extracellular signal-regulated kinase (ERK) 1 and ERK2 in multidrug resistant (MDR) hepatocellular carcinoma (HCC) cells.

METHODS: MDR HCC cell lines, HepG2/adriamycin (ADM) and SMMC7721/ADM, were developed by exposing parental cells to stepwise increasing concentrations of ADM. MTT assay was used to determine drug sensitivity. Flow cytometry was employed to analyze cell cycle distribution and measure cell P-glycoprotein (P-gp) and multidrug resistant protein 1 (MRP1) expression levels. ERK1 and ERK2 mRNA expression levels were measured by quantitative real-time PCR (QRT-PCR). Expression and phosphorylation of ERK1 and ERK2 were analyzed by Western blot.

RESULTS: MTT assay showed that HepG2/ADM and SMMC7721/ADM were resistant not only to ADM, but also to multiple anticancer drugs. The P-gp expression was over 10-fold higher in HepG2/ADM cells than in HepG2 cells (8.92% ± 0.22% *vs* 0.88% ± 0.05%, $P < 0.001$) and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells (7.37% ± 0.26% *vs* 1.74% ± 0.25%, $P < 0.001$). However, the MRP1 expression was not significantly higher in HepG2/ADM and SMMC7721/ADM cells than in parental cells. In addition, the percentage of MDR HepG2/ADM and SMMC7721/ADM cells was significantly decreased in the G0/G1 phase and increased in the the S phase or G2/M phase. QRT-PCR analysis demonstrated that the ERK1 and ERK2 mRNA expression increased apparently in HepG2/ADM cells and decreased significantly in SMMC7721/ADM cells. Compared with the expression of parental cells, ERK1 and ERK2 protein expressions were markedly decreased in SMMC7721/ADM cells. However, ERK2 protein expression was markedly increased while ERK1 protein expression had no significant change in HepG2/ADM cells. Phosphorylation of ERK1 and ERK2 was markedly decreased in both HepG2/ADM and SMMC7721/ADM MDR cells.

CONCLUSION: ERK1 and ERK2 activities are down-regulated in P-gp-mediated MDR HCC cells. ERK1 or ERK2 might be a potential drug target for circumventing MDR HCC cells.

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Key words: Multidrug resistance; Extracellular signal-regulated MAP kinases; Hepatocellular carcinoma; P-glycoprotein; Multidrug resistance-associated protein

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third cause of cancer-related death[1,2]. Drugs used in the treatment of HCC are cytotoxic with a high risk of side effects and none of them is specific for HCC[3]. Moreover, HCC is a hypervascular solid cancer characterized by a high degree of drug resistance[4]. Multidrug resistance (MDR) to chemotherapeutic agents plays a major role in the failure of cancer therapy[5]. MDR phenotype, an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated drugs, is almost constantly expressed in HCC and represents one of the major problems in cancer eradication by limiting the efficacy of chemotherapy[6]. Resistance to therapy can result from decreased drug uptake, increased DNA repair or drug inactivation[7].

Mitogen-activated protein kinase (MAPK) pathway is an attractive target or therapeutic intervention in cancer due to its integral role in the regulation of cancer cell proliferation, invasiveness, and survival. Pharmaceutical agents can inhibit various kinases and GTPases comprising the pathway[8,9]. Extracellular signal-regulated kinase (ERK) 1/2 is a member of the MAPK family. ERK1 and ERK2 are isoforms of the "classical" MAPK[10]. The activity of ERK1/2 has been implicated in the regulation of embryonic morphogenesis, cell proliferation, tumor transformation, and apoptosis[11]. It has been recently found that P-gp expression in the MDR1-transduced human breast cancer cell lines MCF-7/MDR and MDA-MB-231/MDR is positively regulated by the ERK pathway and blockade of the MEK-ERK-RSK pathway can suppress cell surface P-gp expression by promoting its degradation[12]. In addition, there are several lines of evidence that modulation of ERK activation may reverse MDR in prostatic, gastric and hematopoietic cancers[13-16]. However, there is little evidence that ERK activity is related to MDR of HCC.

The aim of this research was to study the crucial kinases of ERK pathway, including expression and phosphorylation (activity) of ERK1 and ERK2 in MDR HCC cell lines, and to explore whether the relationship between MDR and ERK1/2 kinases involves specific molecular aspects of these cell lines. Once they are well characterized, the ERK pathway might be exploited for overcoming MDR of HCC.

MATERIALS AND METHODS

Cell culture

Human HCC cell lines, HepG2 and SMMC7721, were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. HepG2 was cultured with DMEM (HyClone, Logan, UT, USA) and SMMC7721 was cultured with RPMI-1640 (HyClone, Logan, UT, USA). Both media were supplemented with 10% calf serum (HyClone, Logan, UT, USA) and maintained at 37℃ in a humidified atmosphere containing 50 mL/L CO_2 and 950 mL/L air. Multidrug resistant human HCC cell lines, HepG2/adriamycin (ADM) and SMMC7721/ADM, were developed by our group. To develop the HepG2/ADM and SMMC7721/ADM cells, ADM (Pharm-sh Pharmaceutical Co., Ltd., Shanghai, China) was added respectively to HepG2 and SMMC7721 cells at a stepwise increasing concentration from 0.01 to 0.2 mg/L. Resistant cells were selected by removing the non-resistant dead cells. Multidrug resistance was maintained by culturing the cells with 0.2 mg/L ADM and MDR cells were named HepG2/ADM and SMMC7721/ADM.

Measurement of cellular sensitivity to anticancer drugs

MTT (Sigma-Aldrich, St. Louis, MO, USA) assay was used to determine drug sensitivity. Sensitivity of cultured HepG2/ADM and SMMC7721/ADM cells to anticancer drugs, including ADM, fluorouracil, cisplatin, cyclophosphamide, mitomycin and vincristine (Pharm-sh Pharmaceutical Co., Ltd., Shanghai, China), was detected, respectively[17]. IC_{50} value was assessed by probit regression analysis using SPSS11.5 statistical software. Resistance index (RI) was calculated according to the formula: RI = IC_{50} for MDR cells IC_{50}/IC_{50} for parental cells.

Flow cytometric analysis of cell cycle distribution

Cultured HepG2/ADM and SMMC7721/ADM cells and their parental cells were collected respectively through trypsinization, washed with ice-cold PBS, centrifuged at 500 × *g* for 5 min at 4℃, washed twice with ice-cold PBS and fixed in 70% ethanol for 2 h at 4℃. Samples were rehydrated with PBS and the cells were incubated for 30 min at room temperature with a propidium iodide stating solution in PBS containing 0.2 mg/mL propidium iodide, 0.2 mg/mL DNAse-free RNAse A (Roche, Basel, Switzerland), and 0.1% Triton X-100. Using red propidium-DNA fluorescence, 4000 events were acquired with a Epics@ XL Beckman Coulter FACS machine (Beckman Coulter Inc., Fullerton, CA, USA) for each sample and the percentage of cells in G0/G1, S and G2/M phases of the cell cycle was calculated using the Systerm Ⅱ™ software (Beckman Coulter Inc., Fullerton, CA, USA).

Flow cytometric analysis of cell p-gp and mrp1 expression level

Cultured MDR and parental cells were collected as above. Then, samples were immunostained with P-glycoprotein antibody (FITC) (Cat.ab66250, Abcam plc, Cambridge, UK) and MRP1 antibody (FITC) (Cat.No.557593, BD Biosciences Pharmingen, San Diego, CA, USA), respectively, according to the proper protocol. The cells were fixed and permeabilized with a BD Cytofix/Cytoperm™ solution (Cat.No.554722, BD Biosciences, San Jose, CA, USA) before they were immunostained with MRP1 antibody (FITC). Flow cytometry was carried out with a fluorescent-activated cell scan (FACS) using the Systerm Ⅱ™ software. Fluorescence of the cells treated with fluorescent isotype control IgG (Cat.ab18455, Abcam plc) was evaluated in each experiment to measure the level of background fluorescence of negative cells.

Table 1 Determination of IC_{50} and resistance index of different anticancer drugs (mean ± SD)

	IC_{50}		Resistance index	IC_{50}		Resistance index
	HepG2	HepG2/ADM		SMMC7721	SMMC7721/ADM	
Adriamycin (mg/L)	0.0063 ± 0.0022	0.135 ± 0.053	21.43	0.0139 ± 0.008	0.266 ± 0.036	19.14
Fluorouracil (μmol/L)	1.114 ± 0.271	25.34 ± 2.38	22.75	0.689 ± 0.082	48.5 ± 2.57	70.39
Cyclophosphamide (mg/L)	2.902 ± 0.369	32.68 ± 4.962	11.26	2.315 ± 0.279	60.08 ± 4.93	25.95
Cisplatin (mg/L)	0.0527 ± 0.013	1.084 ± 0.0749	20.57	0.0483 ± 0.011	1.637 ± 0.172	33.89
Mitomycin (mg/L)	0.061 ± 0.017	1.085 ± 0.246	17.79	0.032 ± 0.013	0.644 ± 0.168	20.13
Vincristine (mg/L)	0.0093 ± 0.0035	0.086 ± 0.0098	9.25	0.006 ± 0.004	0.247 ± 0.023	41.16

Mean fluorescence intensity (MFI) of positively stained cells was determined.

RNA extraction and quantitative real-time polymerase chain reactions (QRT-PCR)

ERK1 and ERK2 mRNA expression levels were measured by QRT-PCR. Total RNA was extracted using the TRIzol reagent (GIBCO BRL, Life Technologies Inc., Rockville, MD, USA) following the constructions of its manufacturer, and reverse transcripted to cDNA using the Gene Amp RNA PCR kit in a DNA thermal cycler (Bio-Rad). QRT-PCR was performed with SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 7700 real time PCR machine (Applied Biosystems).

The synthesized cDNA served as a template in a (25 μL) reaction. A non-template control was included in all experiments. Primer sequences are as follows: ERK1 GenBank: NM_002746 forward, 5'-TCAACAC CACCTGCGACCTT-3', and reverse, 5'-GCGTAGCC ACATACTCCGTCA-3'; ERK2 GenBank: NM_002745 forward, 5'-GTTCCCAAATGCTGACTCCAA-3', and reverse, 5'-CTCGGGTCGTAATACTGCTCC-3'; β-actin GenBank: NM_001101 forward, 5'-TGACG TGGACATCCGCAAAG-3', and reverse, 5'-CTG-GAAGGTGGACAGCGAGG-3'.

QRT-PCR was performed at 94℃ for 4 min, followed by 40 cycles at 94℃ for 15 s, at 60℃ for 25 s, and at 72℃ for 25 s. Oligonucleotides and reagents for PCR assay were purchased from Qiagen GmbH, Hilden, Germany. Data were analyzed with the Sequence detector software (v1.9, Applied Biosystems). The mean Ct value for duplicate measurements was used to detect the expression of target gene with normalization to a housekeeping gene used as an internal control (β-actin) according to the $2^{-\Delta Ct}$ formula.

Western blot analysis

Protein was collected from cultured HepG2, SMMC7721, HepG2/ADM and SMMC7721/ADM cells and its concentration was measured (protein assay dye, Bio-Rad). Then, the protein was denatured in a LDS sample buffer for 5 min at 95℃, run on SDS-PAGE (NUPAGE, 4%-12% Bis-Tris, Invitrogen, Carlsbad, CA, USA) and blotted onto PVDF membranes (0.2 μm, Invitrogen). Membranes were blocked with 5% dry milk in TBS-T (TBS containing 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4℃ with antibodies against ERK1, ERK2, or phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Inc., Danvers, MA, USA). After incubation with the respective primary antibodies, the membranes were exposed to species-specific horseradish peroxidase-labeled secondary antibodies at room temperature, and developed using the ECL plus Western blotting reagent (GE Healthcare, Little Chalfont, UK) and Fuji Film LAS-1000 equipment (Fuji Film, Tokyo, Japan). Parallel membranes were incubated with 1:5000 rabbit monoclonal antibodies to GAPDH (Cell Signaling Technology, Inc.) and HRP-coupled rabbit anti-mouse secondary antibody. Primary and secondary antibody solutions were prepared in a PBS solution containing 2% bovine serum albumin and 0.1% Tween-20. After incubation with antibodies, the membranes were washed 3 times for 5 min in PBS containing 0.1% Tween-20. Calculation and statistics were performed using the ImageJ 1.37 software.

Statistical analysis

Statistical analysis was performed using Student's *t*-test to compare the two groups and ANOVA was used with Dunnett's post-test for multiple comparisons when the three groups or more were compared. $P < 0.05$ was considered statistically significant. The results were expressed as mean ± SE. Values were analyzed using the statistical package SPSS for Windows Ver.11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Determination of MDR

Each step of developing MDR HepG2/ADM and SMMC7721/ADM cells took 7-8 wk. MDR was maintained by culturing the cells with 0.2mg/L ADM. Cytotoxicity assay found that HepG2/ADM and SMMC7721/ADM were resistant not only to ADM but also to multiple anticancer drugs. Among them, fluorouracil (5-FU), cyclophosphamide (CTX), cisplatin (CDDP), mitomycin (MMC), and vincristine (VCR) were tested in our study. Their lethal dose (IC_{50}) was significantly higher for HepG2/ADM and SMMC7721/ADM cells than for non-resistant parental cells (Figure 1 and Table 1).

Cell cycle distribution

Cell cycle phase distribution was detected by flow cytometry to determine whether there is any difference in cell cycle kinetics between MDR HepG2/ADM and SMMC7721/

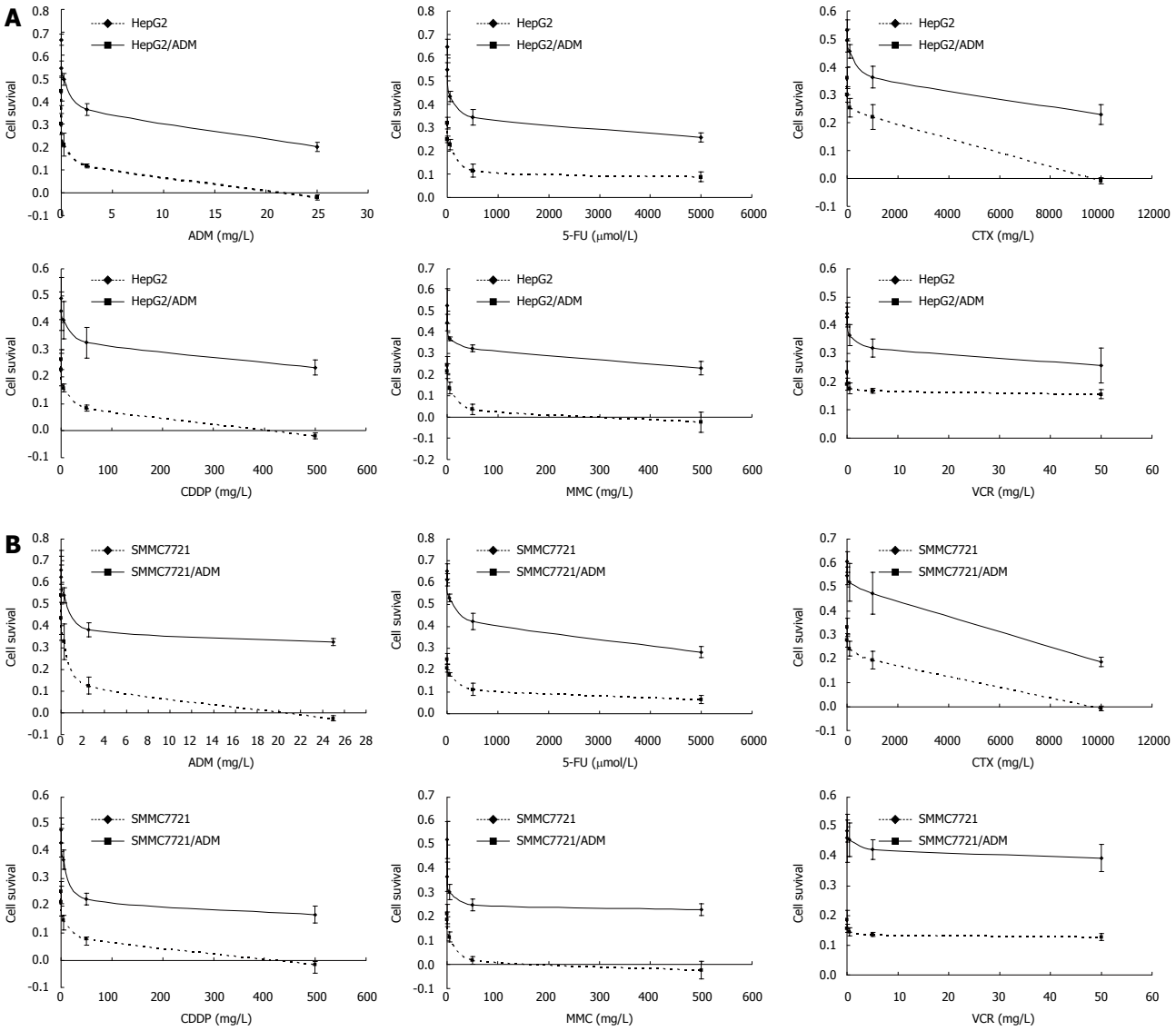

Figure 1 Measurement of cellular sensitivity to anticancer drugs and parental cells. Cytotoxicity assay for adriamycin, fluorouracil, cyclophosphamide, tested cisplatin, mitomycin, and vincristine was performed to evaluate the IC50 for HepG2 and HepG2/ADM cells (A), SMMC7721 and SMMC7721/ADM cells (B). Dose response curves were derived from five independent experiments using MTT assay. The data were shown as mean ± SE.

Table 2 Cell cycle distribution of parental and MDR HCC cells (mean ± SD)

Cells (n = 5)	G0/G1	S	G2/M
hepG2	65.08 ± 1.61	18.32 ± 1.37	16.58 ± 0.65
hepG2/ADM	62.44 ± 1.77[a]	12.24 ± 1.21[b]	25.36 ± 2.12[c]
SMMC7721	71.12 ± 1.38	17.86 ± 1.91	11.02 ± 1.95
SMMC7721/ADM	67.8 ± 2.15[a]	23.6 ± 0.93[b]	8.62 ± 2.74[a]

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs* control cells.

Table 3 P-gp and MRP1 expression in MDR and parental cells (mean ± SD)

Cells (n = 5)	P-gp (%)	MRP1 (%)
HepG2	0.88 ± 0.05	0.93 ± 0.15
HepG2/ADM	8.92 ± 0.22[a]	0.9 ± 0.18
SMMC7721	1.74 ± 0.25	1.21 ± 0.35
SMMC7721/ADM	7.37 ± 0.26[a]	0.79 ± 0.02

[a]$P < 0.001$ *vs* parental cells.

ADM cells and their parental cells. The percentage of HepG2/ADM and SMMC7721/ADM cells was significantly decreased at the G0/G1 phase and increased at the S phase or G2/M phase (Figure 2 and Table 2).

P-gp and MRP1 expression

Protein expression of P-gp and MRP1 in HepG2/ADM, SMMC7721/ADM and their parental cells was evaluated. P-gp expression was over 10-fold higher in HepG2/ADM cells than in HepG2 cells, and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells. However, the MRP1 expression was not significantly higher in HepG2/ADM and SMMC7721/ADM cells than in parental cells (Figure 3 and Table 3).

ERK1 and ERK2 mRNA expression

To examine the role of ERK signaling pathway in the development of MDR, ERK1 and ERK2 mRNA

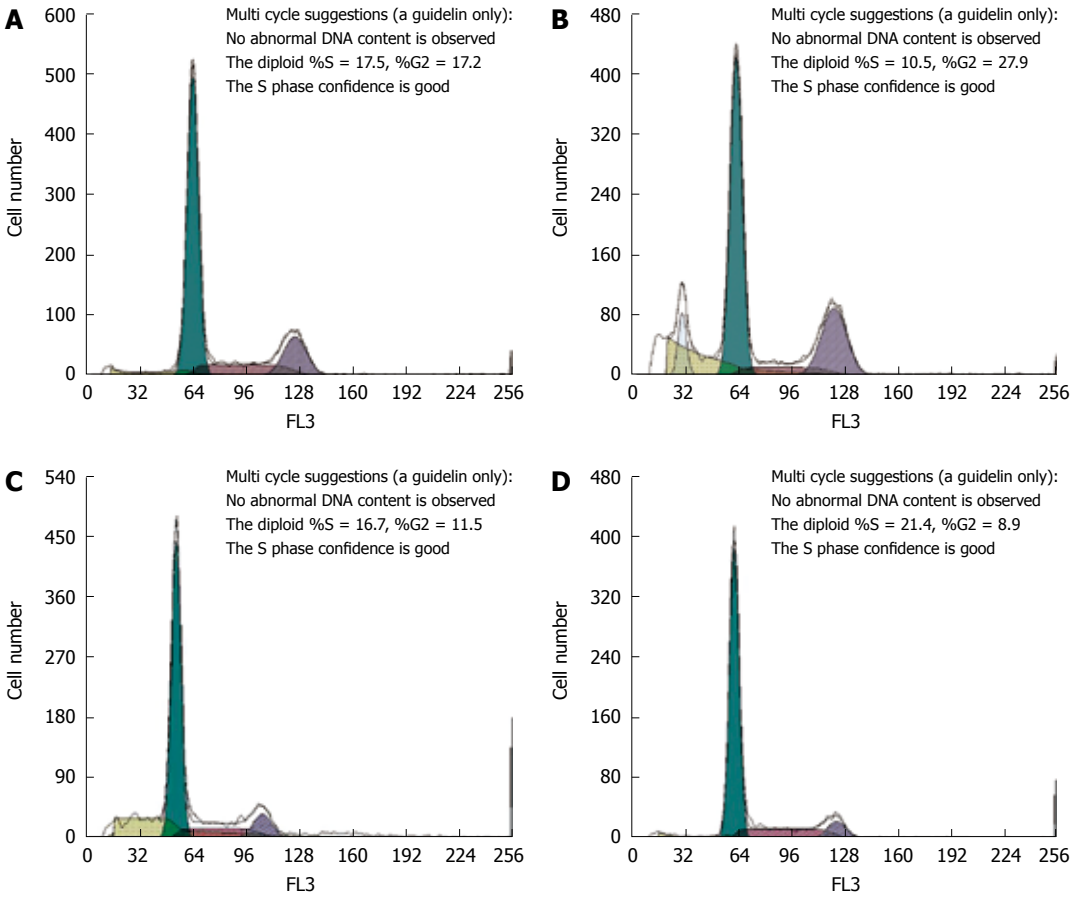

Figure 2 Cell cycle distributions in MDR and parental cells. Cell cycle distributions of HepG2 (A), HepG2/ADM (B), SMMC7721 (C) and SMMC7721/ADM (D) cells were analyzed by flow cytometry as described in Materials and Methods.

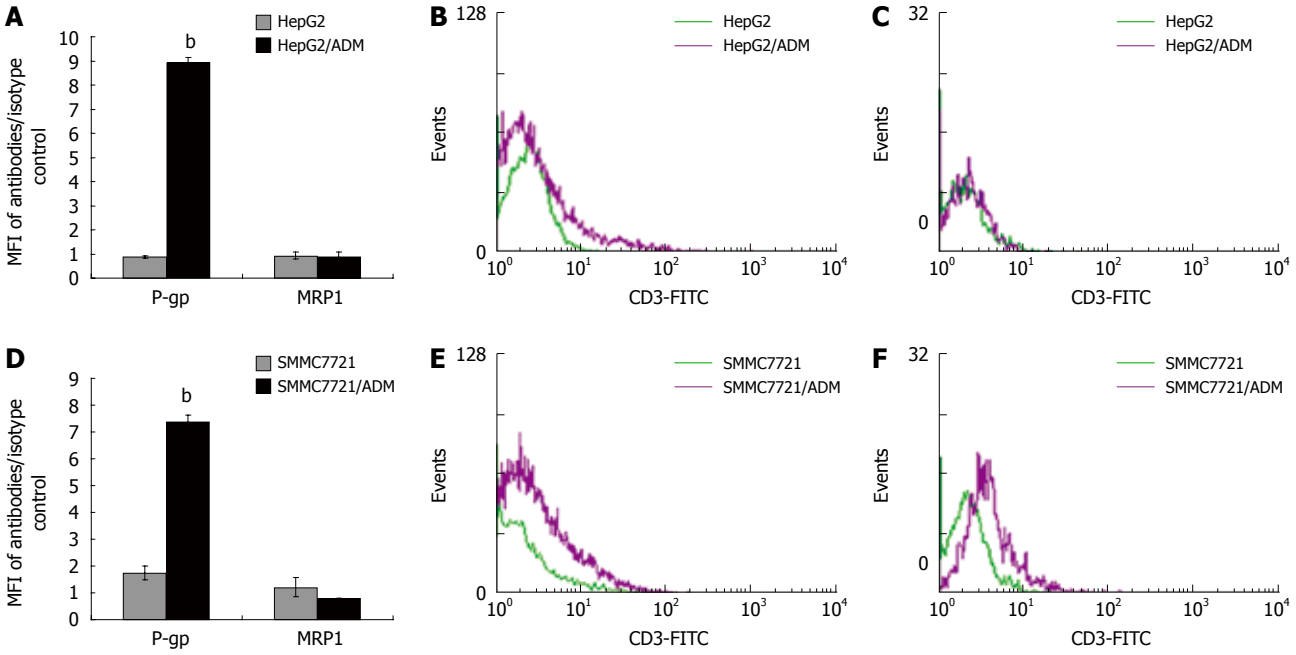

Figure 3 Expression of P-gp and MRP1 in MDR and parental cells. Histograms showing that P-gp expression was over 10-fold higher in HepG2/ADM cells than in HepG2 cells, and over 4-fold higher in SMMC7721/ADM cells than in SMMC7721 cells, but MRP1 expression had no significant difference (A, D). Corresponding flow histograms for P-gp (B, E) and MRP1 (C, F) are presented. The results are shown as mean ± SE (n = 5). Statistical analyses comparing MDR cells with parental cells were performed using Student's t-test. $^bP < 0.001$ *vs* SMMC7721 cells.

expression in parental and MDR cells was assessed, respectively (Figure 4). QRT-PCR analysis demonstrated that ERK1 and ERK2 mRNA expression was apparently higher in HepG2/ADM cells and significantly lower in SMMC7721/ADM cells.

Expression and phosphorylation of ERK1 and ERK2

The expression of ERK1, ERK2, p-ERK1 and p- ERK2 in HepG2/ADM, SMMC7721/ADM and their parental cells, was detected by Western blot analysis, respectively. The expression of ERK1 and ERK2 protein was

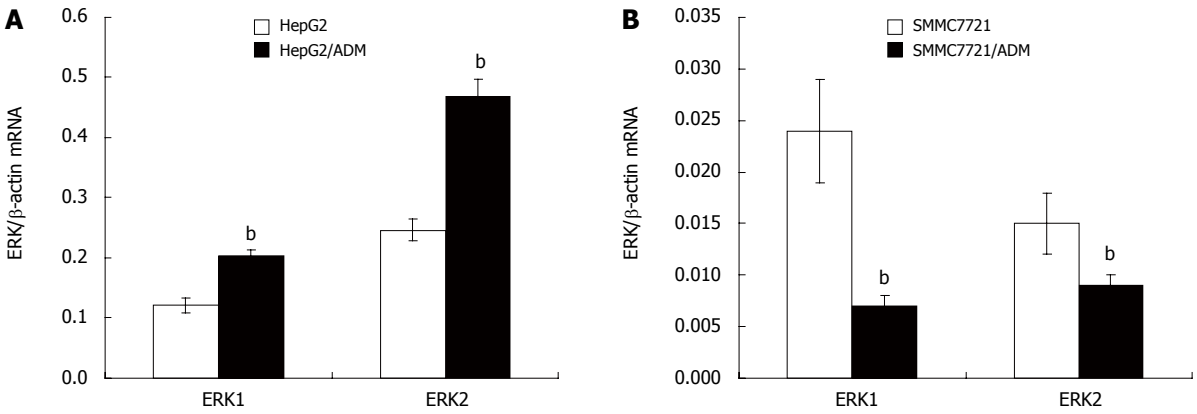

Figure 4 ERK1 and ERK2 mRNA expression in MDR and parental cells. ERK1 and ERK2 mRNA levels were measured by QRT-PCR. A: HepG2/ADM and HepG2 cells; B: SMMC7721/ADM and SMMC7721 cells. Results were normalized by β-actin mRNA expression and compared with the levels in parental cells (n = 3). The results are shown as mean ± SE. Statistical analyses comparing MDR cells with parental cells were performed using Student's t-test. $^{b}P < 0.01$ *vs* parental cells (data not shown).

markedly lower in SMMC7721/ADM cells than in their parental cells. However, the expression of ERK2 protein was significantly higher in SMMC7721/ADM cells than in their parental cells while the expression of ERK1 protein had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was markedly lower in HepG2/ADM and SMMC7721/ADM MDR cells than in their parental cells (Figure 5).

DISCUSSION

Multidrug resistant cancer cells may develop in patients upon prolonged treatment with anti-cancer drugs. MDR poses a great obstacle to chemotherapy for cancer because a higher dosage of drugs is needed to be administered to patients, which will cause severe adverse effects[18]. Most strategies developed to reverse MDR phenotype involve use of resistance modulators, which have the common ability to reverse the phenotype by inhibiting MDR transporter function[19,20]. A more efficient strategy to circumvent MDR is to down-regulate the expression of genes coding transporters. However, regulation of MDR-related gene expression is highly complex. For instance, such complexity is embodied in multiple transcription-regulatory elements in the 5' and 3' flanking sequences of the mdr-1 gene, and numerous protein factors involved in transcription-regulatory processes in a cell type- and stimulus-dependent manner[21]. The mechanism underlying the expression of MDR-related genes has not yet been fully understood. Thus, the molecular mechanism and signal-transduction pathways involved in regulation of MDR-related genes should be further studied in order to overcome MDR and improve chemotherapeutic efficacy.

The ERK signaling pathway mediates a number of cellular processes, including cell differentiation, growth, survival, and apoptosis. Several growth factors stimulate a protein kinase cascade that sequentially activates Raf, MEK, and ERK1/2. The role of ERK pathway in the generation of MDR has received more consideration than before, and some specific blocking agents of the ERK pathway have been found. Recent studies demonstrated that modulation of ERK activation may be a new method to reverse MDR[15,22]. However, whether ERK activation is positively or negatively correlated with MDR still remains controversial. Furthermore, the relationship between ERK activity and MDR of HCC is unknown. In this study, we found that ERK activity was down-regulated in MDR HCC Cells.

ADM is a chemotherapeutic agent principally used in treatment of solid tumors, including HCC[23]. Although ADM can work through various mechanisms, it is still not immune to the MDR phenotype, and consequently, development of resistance to ADM has been well-documented in a broad range of cell lines[24-26]. We established two MDR HCC cell lines, HepG2/ADM and SMMC7721/ADM. In this study, death of MDR cells with a lower drug resistance occurred at about 24-48 h after a higher concentration of ADM was added in the medium, and reached the peak at about 72 h. To recover the proliferation ability, the culture of remained adherent cells took at least 7-8 wk. The whole development of MDR HCC cell lines lasted ten months. The IC_{50} of anticancer drugs was much higher for HepG2/ADM and SMMC7721/ADM cells than for their parental cells, suggesting that the acquired MDR of HepG2/ADM and SMMC7721/ADM is reliable.

To elucidate the mechanism involved in the development of acquired MDR of HepG2/ADM and SMMC7721/ADM cells, cell cycle distribution and expression of MDR-related proteins (P-gp and MRP1) were analyzed by FCM. The percentage of MDR HepG2/ADM and SMMC7721/ADM cells was significantly decreased in the G0/G1 phase and increased in the S phase or G2/M phase than that of their parental cells, which probably contributes to the lower ability of cells to proliferate. Moreover, this kind of delayed cell cycle can result in cellular escape of cytotoxicity of cell cycle specific agents (e.g. vincristine, fluorouracil, *etc.*) and generate MDR[27,28]. P-gp and MRP1 are members of the ATP-binding cassette transporter proteins. Over-expression of ATP-binding cassette transporter proteins

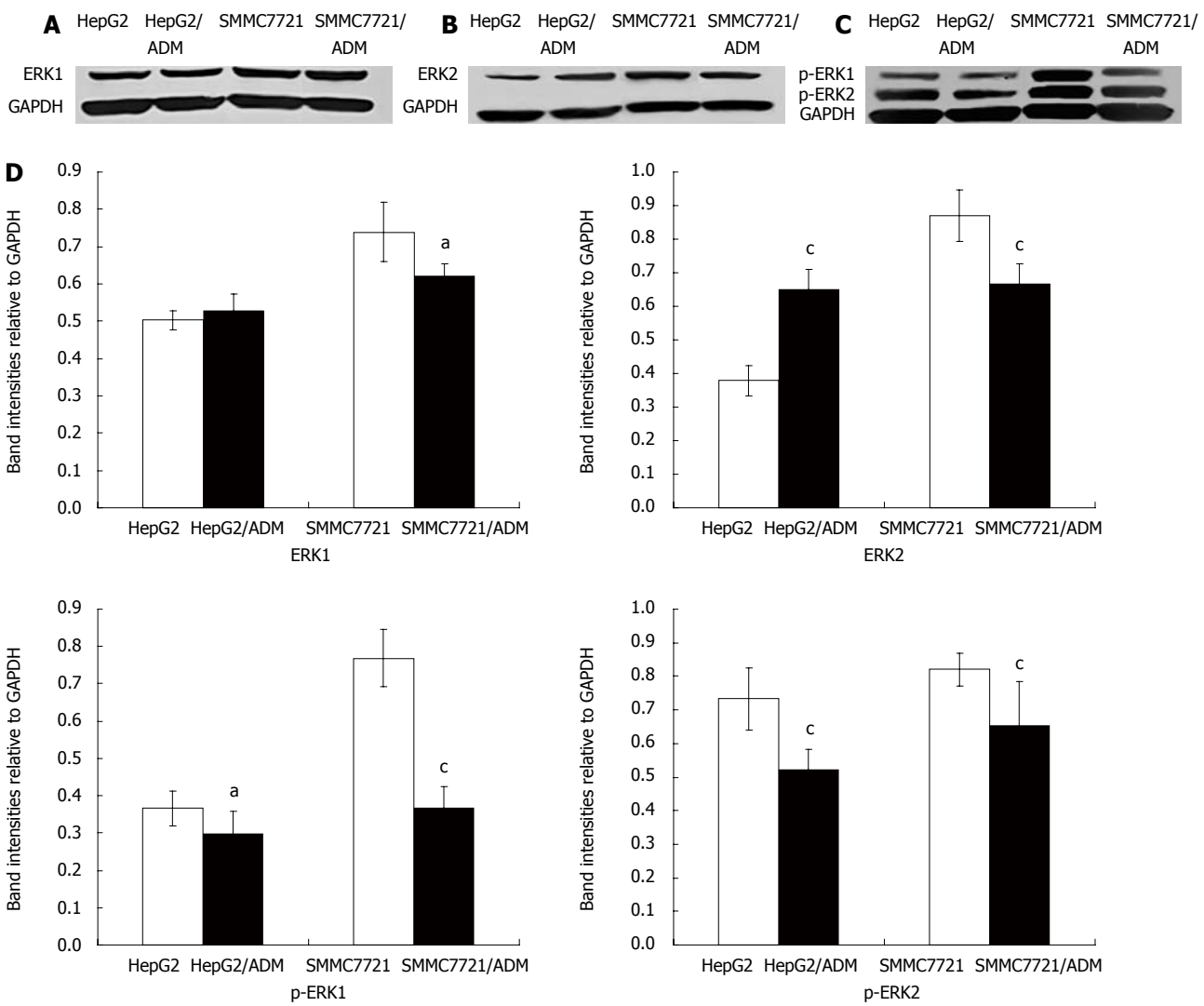

Figure 5 Expression and phosphorylation of ERK1 and ERK2 in MDR and parental cells. Western blot analysis of the ERK1 (A), ERK2 (B), p-ERK1 and p-ERK2 (C) expression in HepG2/ADM, SMMC7721/ADM as well as in HepG2 and SMMC7721 cells (n = 3) was performed. The expression of ERK1 and ERK2 was markedly lower in SMMC7721/ADM cells than in parental cells. However, the ERK2 expression was markedly increased and the ERK1 expression had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was lower in MDR cells than in parental cells (D). The results are shown as mean ± SE. Statistical analyses comparing MDR cells with parental cells were performed using Student's t-test. $^{a}P < 0.05$, $^{c}P < 0.01$ *vs* parental cells (data not shown).

represents one of the major mechanisms that contribute to the MDR phenotype. Both P-gp and MRP1 function as a drug efflux pump that actively transports drugs from the inside to the outside of cells and causes a defect in the intracellular accumulation of drugs necessary for cancer cell killing. The results of our study show that P-gp was much higher in MDR HepG2/ADM and SMMC7721/ADM cells than in their parental cells, but the expression of MRP1 was low in both MDR and parental cells, indicating that MDR of HepG2/ADM and SMMC7721/ADM cells mainly attribute to the over-expression of P-gp but not MRP1. This phenomenon can partially be explained by the high expression of P-gp and low expression of MRP1 in liver tissue or HCC cell lines[29].

ERK1 and ERK2, isozymes of ERK, are extensively expressed in cultured cell lines and mammalian tissues[30]. To answer the question of whether ERK1 and ERK2 are involved in P-gp-mediated MDR in HCC cells, we detected the expression of ERK1 and ERK2 mRNA in parental and MDR cells, and the expression and phosphorylation (activity) of ERK1 and ERK2 protein. The results showed that the expression of ERK1 and ERK2 mRNA was increased in HepG2/ADM cells and decreased in SMMC7721/ADM cells. However, ERK1 protein expression had no significant change in HepG2/ADM cells. The phosphorylation of ERK1 and ERK2 was markedly decreased in HepG2/ADM and SMMC7721/ADM MDR cells, suggesting that ERK1 and ERK2 activity is down-regulated in P-gp-mediated MDR HCC cells. However, the decreased activity was not in accordance with mRNA and protein expression in HepG2/ADM cells. The expression of ERK1 and ERK2 protein was diverse, which may contribute to the augments on whether ERK activation is positively or negatively correlated with MDR.

In summary, MDR of HepG2/ADM and SMMC7721/ADM cells mainly attribute to the over-expression of P-gp but not MRP1. ERK1 and ERK2

activity is down-regulated in P-gp-mediated MDR HCC cells, providing new insights into the complicated regulatory mechanism of MDR phenotype. ERK1 and ERK2 might be potential drug targets for circumventing MDR HCC cells. *In vivo* studies are warranted to examine whether ERK1 and ERK2 have a clinical potential in modulating the MDR phenotype during HCC chemotherapy.

COMMENTS

Background

The development of multidrug resistance (MDR) to chemotherapeutic agents plays a major role in the failure of cancer therapy, including hepatocellular carcinoma (HCC). Recent studies have shown that modulation of extracellular signal-regulated kinase (ERK) activation may reverse MDR in prostatic, gastric and hematopoietic cancers. However, there is little evidence that ERK activity is related with MDR of HCC cells.

Research frontiers

Multidrug resistant cancer cells may develop in patients upon prolonged treatment with anti-cancer drugs. Most strategies developed to reverse the MDR phenotype involve use of resistance modulators. A more efficient strategy to circumvent MDR is to down-regulate the expression of genes coding for transporters. Thus, to overcome MDR and improve chemotherapeutic efficacy, the molecular mechanism and signal-transduction pathway involved in the regulation of MDR-related genes should be further studied.

Innovations and breakthroughs

In this study, the MDR of HepG2/ADM and SMMC7721/ADM cells could attribute to the over-expression of P-gp but not MRP1, ERK1 and ERK2 activity was down-regulated in P-gp-mediated MDR HCC cells, thus providing new insights into the complicated regulatory mechanism of MDR phenotype.

Applications

ERK1 and ERK2 might be used as potential drugs targets for circumventing HCC MDR.

Terminology

Multidrug resistance: an intrinsic or acquired cross-resistance to a variety of structurally and functionally unrelated drugs, which is almost constantly expressed in cancer and represents one of the major problems in cancer eradication by limiting the efficacy of chemotherapy, and resistance to therapy can result from decreased drug uptake, increased DNA repair or drug inactivation.

Peer review

The authors examined the expression and phosphorylation of ERK1/2 in MDR HCC cell lines, and demonstrated that ERK1 and ERK2 activity was down-regulated in P-gp-mediated MDR HCC cells, indicating that ERK1 or ERK2 might be used as a potential drug target for circumventing MDR HCC cells.

REFERENCES

1 **Okuda K**. Hepatocellular carcinoma. *J Hepatol* 2000; **32**: 225-237
2 **Parkin DM**, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153-156
3 **Avila MA**, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. *Oncogene* 2006; **25**: 3866-3884
4 **Wakamatsu T**, Nakahashi Y, Hachimine D, Seki T, Okazaki K. The combination of glycyrrhizin and lamivudine can reverse the cisplatin resistance in hepatocellular carcinoma cells through inhibition of multidrug resistance-associated proteins. *Int J Oncol* 2007; **31**: 1465-1472
5 **Perez-Tomas R**. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr Med Chem* 2006; **13**: 1859-1876
6 **Folmer Y**, Schneider M, Blum HE, Hafkemeyer P. Reversal of drug resistance of hepatocellular carcinoma cells by adenoviral delivery of anti-ABCC2 antisense constructs. *Cancer Gene Ther* 2007; **14**: 875-884
7 **Modok S**, Mellor HR, Callaghan R. Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. *Curr Opin Pharmacol* 2006; **6**: 350-354
8 **Panka DJ**, Atkins MB, Mier JW. Targeting the mitogen-activated protein kinase pathway in the treatment of malignant melanoma. *Clin Cancer Res* 2006; **12**: 2371s-2375s
9 **Johnson GL**, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002; **298**: 1911-1912
10 **Lewis TS**, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998; **74**: 49-139
11 **Avruch J**. MAP kinase pathways: the first twenty years. *Biochim Biophys Acta* 2007; **1773**: 1150-1160
12 **Katayama K**, Yoshioka S, Tsukahara S, Mitsuhashi J, Sugimoto Y. Inhibition of the mitogen-activated protein kinase pathway results in the down-regulation of P-glycoprotein. *Mol Cancer Ther* 2007; **6**: 2092-2102
13 **Kisucka J**, Barancik M, Bohacova V, Breier A. Reversal effect of specific inhibitors of extracellular-signal regulated protein kinase pathway on P-glycoprotein mediated vincristine resistance of L1210 cells. *Gen Physiol Biophys* 2001; **20**: 439-444
14 **Lin JC**, Chang SY, Hsieh DS, Lee CF, Yu DS. Modulation of mitogen-activated protein kinase cascades by differentiation-1 protein: acquired drug resistance of hormone independent prostate cancer cells. *J Urol* 2005; **174**: 2022-2026
15 **Li Y**, Li S, Han Y, Liu J, Zhang J, Li F, Wang Y, Liu X, Yao L. Calebin-A induces apoptosis and modulates MAPK family activity in drug resistant human gastric cancer cells. *Eur J Pharmacol* 2008; **591**: 252-258
16 **McCubrey JA**, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D' Assoro AB, Salisbury JL, Mazzarino MC, Stivala F, Libra M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 2006; **46**: 249-279
17 **Chen B**, Sun Q, Wang X, Gao F, Dai Y, Yin Y, Ding J, Gao C, Cheng J, Li J, Sun X, Chen N, Xu W, Shen H, Liu D. Reversal in multidrug resistance by magnetic nanoparticle of Fe3O4 loaded with adriamycin and tetrandrine in K562/A02 leukemic cells. *Int J Nanomedicine* 2008; **3**: 277-286
18 **Stavrovskaya AA**. Cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry* (Mosc) 2000; **65**: 95-106
19 **Angelini A**, Iezzi M, Di Febbo C, Di Ilio C, Cuccurullo F, Porreca E. Reversal of P-glycoprotein-mediated multidrug resistance in human sarcoma MES-SA/Dx-5 cells by nonsteroidal anti-inflammatory drugs. *Oncol Rep* 2008; **20**: 731-735
20 **Yan F**, Jiang Y, Li YM, Zhen X, Cen J, Fang WR. Reversal of P-glycoprotein and multidrug resistance-associated protein 1 mediated multidrug resistance in cancer cells by HZ08 Isomers, tetrataisohydroquinolin derivatives. *Biol Pharm Bull* 2008; **31**: 1258-1264
21 **Stierle V**, Duca M, Halby L, Senamaud-Beaufort C, Capobianco ML, Laigle A, Jolles B, Arimondo PB. Targeting MDR1 gene: synthesis and cellular study of modified daunomycin-triplex-forming oligonucleotide conjugates able to inhibit gene expression in resistant cell lines. *Mol Pharmacol* 2008; **73**: 1568-1577
22 **Hu Y**, Bally M, Dragowska WH, Mayer L. Inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase enhances chemotherapeutic effects on H460 human non-small cell lung cancer cells through activation of apoptosis. *Mol Cancer Ther* 2003; **2**: 641-649
23 **Minotti G**, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* 2004; **56**: 185-229
24 **Barrand MA**, Heppell-Parton AC, Wright KA, Rabbitts PH, Twentyman PR. A 190-kilodalton protein overexpressed in

non-P-glycoprotein-containing multidrug-resistant cells and its relationship to the MRP gene. *J Natl Cancer Inst* 1994; **86**: 110-117

25 **Mehta K**. High levels of transglutaminase expression in doxorubicin-resistant human breast carcinoma cells. *Int J Cancer* 1994; **58**: 400-406

26 **Shen DW**, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, Pastan I, Gottesman MM. Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. *J Biol Chem* 1986; **261**: 7762-7770

27 **Rebbaa A**. Targeting senescence pathways to reverse drug resistance in cancer. *Cancer Lett* 2005; **219**: 1-13

28 **Schmitt CA**. Cellular senescence and cancer treatment. *Biochim Biophys Acta* 2007; **1775**: 5-20

29 **Sharom FJ**. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* 2008; **9**: 105-127

30 **Sugden PH**, Clerk A. Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal* 1997; **9**: 337-351

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ORIGINAL ARTICLES

Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To investigate the protective effects of melatonin on carbon tetrachloride (CCl_4)-induced hepatic fibrosis in experimental rats.

METHODS: All rats were randomly divided into normal control group, model control group treated with CCl_4 for 12 wk, CCl_4 + NAC group treated with CCl_4 + NAC (100 mg/kg, i.p.) for 12 wk, CCl_4 + MEL-1 group treated with CCl_4 + melatonin (2.5 mg/kg) for 12 wk, CCl_4 + MEL-2 group treated with CCl_4 + melatonin (5.0 mg/kg) for 12 wk, and CCl_4 + MEL-3 group treated with CCl_4 + melatonin (10 mg/kg). Rats in the treatment groups were injected subcutaneously with sterile CCl_4 (3 mL/kg, body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in treatment groups. At the end of experiment, rats in each group were anesthetized and sacrificed. Hematoxylin and eosin (HE) staining and Van Gieson staining were used to examine changes in liver pathology. Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were measured with routine laboratory methods using an autoanalyzer. Hydroxyproline (HYP) content in liver and malondialdehyde (MDA) and glutathione peroxidase (GPx) levels in liver homogenates were assayed by spectrophotometry. Serum hyaluronic acid (HA), laminin (LN), and procollagen Ⅲ N-terminal peptide (PⅢNP) were determined by radioimmunoassay.

RESULTS: Pathologic grading showed that the fibrogenesis was much less severe in CCl_4 + MEL3 group than in model control group (u = 2.172, P < 0.05), indicating that melatonin (10 mg/kg) can significantly ameliorate CCl_4-induced hepatic fibrotic changes. The serum levels of ALT and AST were markedly lower in CCl_4 + MEL treatment groups (5, 10 mg/kg) than in model control group (ALT: 286.23 ± 121.91 U/L *vs* 201.15 ± 101.16 U/L and 178.67 ± 103.14 U/L, P = 0.028, P = 0.007; AST: 431.00 ± 166.35 U/L *vs* 321.23 ± 162.48 U/L and 292.42 ± 126.23 U/L, P = 0.043, P = 0.013). Similarly, the serum laminin (LN) and hyaluronic acid (HA) levels and hydroxyproline (HYP) contents in liver were significantly lower in CCl_4 + MEL-3 group (10 mg/kg) than in model control group (LN: 45.89 ± 11.71 μg/L *vs* 55.26 ± 12.30 μg/L, P = 0.012; HA: 135.71 ± 76.03 μg/L *vs* 201.10 ± 68.46 μg/L, P = 0.020; HYP: 0.42 ± 0.08 mg/g tissue *vs* 0.51 ± 0.07 mg/g tissue, P = 0.012). Moreover, treatment with melatonin (5, 10 mg/kg) significantly reduced the MDA content and increased the GPx activity in liver homogenates compared with model control group (MDA: 7.89 ± 1.49 noml/mg prot *vs* 6.29 ± 1.42 noml/mg prot and 6.25 ± 2.27 noml/mg prot, respectively, P = 0.015, P = 0.015; GPx: 49.13 ± 8.72 U/mg prot *vs* 57.38 ± 7.65 U/mg prot and 61.39 ± 13.15 U/mg prot, respectively, P = 0.035, P = 0.003).

CONCLUSION: Melatonin can ameliorate CCl_4 -induced hepatic fibrosis in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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Key words: Melatonin; Hepatic fibrosis; Oxidative stress; Hyaluronic acid; Laminin; Malondialdehyde; Glutathione peroxidase

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INTRODUCTION

Hepatic fibrosis, a common pathological process of chronic hepatic disease, can lead to irreversible cirrhosis, and involves multiple cellular and molecular events that ultimately result in accumulation of collagen and extra cellular matrix protein in space of Disse. If treated properly at fibrosis stage, cirrhosis can be prevented[1]. However, no effective antifibrosis drugs are available at present. Several lines of evidence suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis[2,3].

Melatonin (N-acetyl-5-metyoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant, regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger[4], eliminates oxygen free radicals and reactive intermediates[5-9]. Both *in vitro* and *in vivo* experiments have shown that melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes, such as safrole, lipopolysaccharide (LPS), carbon tetrachloride (CCl_4), ischemia-reperfusion, amyloid-protein, and ionizing radiation[10-12]. In addition, melatonin also has an indirect antioxidant effect by enhancing the levels of potential antioxidants such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH)[10-12]. Recent studies showed that melatonin exerts its cytoprotective effects in various experimental models of acute liver injury and reduces fibroblast proliferation and collagen synthesis[12,13], indicating that melatonin may have therapeutic effects on acute and chronic liver injury, through its antioxidant action.

The aim of our present study was to evaluate the possible antifibrotic effect of melatonin on a hepatic fibrosis model of rats. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with liver fibrosis.

MATERIALS AND METHODS

Drugs and materials

Crystalline melatonin was purchased from Sigma Chemical Company (St. Louis, MO, USA). The solvent used for melatonin was a mixture of ethanol (1%, v/v) and NaCl (0.9%). N-acetyl-L-cysteine (NAC) was purchased from Shanghai Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Commercial kits used for determining malondialdehyde (MDA), glutathione peroxidase (GPx) and hydroxyproline (HYP) were obtained from Jiancheng Institute of Biotechnology (Nanjing, China). Commercial kits for radioimmunoassay of procollagen Ⅲ N-terminal peptide (PⅢNP), laminin (LN), and hyaluronic acid (HA) were obtained from Beijing North Institute of Biological Technology (Beijing, China). Other commercial chemicals used in experiments were of analytical grade.

Animal experiments and drug treatment

Male Sprague-Dawley rats, weighing 170-240 g at beginning of the study, purchased from Anli Experimental Animal Limited Company (Anhui, China), were kept at a constant temperature (22℃) in a 12-h light and dark cycle, with free access to food and water. All animals were treated humanely according to the National Guidelines for the Care of Animals in China. Rats were randomly divided into normal control group (n = 11), model control group (n = 20) treated with CCl_4 for 12 wk, CCl_4 + NAC group (n = 20) treated with CCl_4 + NAC (100 mg/kg, i.p.) for 12 wk, CCl_4 + MEL-1 group (n = 20) treated with CCl_4 + melatonin (2.5 mg/kg) for 12 wk, CCl_4 + MEL-2 group (n = 20) treated with CCl_4 + melatonin (5.0 mg/kg) for 12 wk, and CCl_4 + MEL-3 group (n = 20) treated with CCl_4 + melatonin (10 mg/kg) for 12 wk. Rats in treatment groups were injected subcutaneously with sterile CCl_4 (3 mL/kg of body weight) in a ratio of 2:3 with olive oil twice a week. Rats in normal control group received hypodermic injection of olive oil at the same dose and frequency as those in the treatment groups. At the beginning of CCl_4 injection, rats received intraperitoneal melatonin daily whereas rats that did not receive melatonin were given the same volume of vehicle (1% ethanol) at the same time point. After 12 wk, a laparotomy was performed and blood was drawn from the abdominal aorta under 3% pentobarbital sodium (1 mL/kg) anesthesia. The animals were then killed with their livers removed. Blood was collected into tubes and centrifuged. Serum was aspirated and stored at -80℃. Liver tissue was fixed in formalin for routine histological examination, or stored at -80℃ until required.

Histopathological examination

Liver tissue samples, fixed in 40 g/L paraformaldehyde and embedded in paraffin, were cut into 5-μm thick sections, which were stained with hematoxylin and eosin (HE) and Van Gieson (VG) according to the standard procedure. Van Gieson's method was used to detect collagen fibers. Hepatic fibrosis was divided into the following stages as previously described[14]: stage 0: no fibrosis; stage 1: expansion of portal tracts without linkage; stage 2: portal expansion with portal to portal linkage; stage 3: expansive portal to portal and focal portal to central linkage; and stage 4: cirrhosis. Two pathologists with no knowledge of liver sources examined the stained sections independently.

Analysis of liver function

Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and protein concentration were measured with routine laboratory methods using an autoanalyzer (Hitachi Automatic Analyzer, Japan).

Measurement of MDA and GPx levels in liver homogenates

Liver samples were thawed, weighed and homogenized (1:9 w:v) in 0.9% saline. The homogenates were centrifuged at 1000 × *g* for 10 min at 4℃ and supernatant was taken for assay of MDA and GPx with a commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer's instructions. MDA was assayed by measuring the levels of thiobarbituric acid reactive substances (TBARS) at 532 nm and expressed as nmol/mg protein. GPx assay was based on its ability to inhibit oxidation of oxyamine by the xanthine-xanthine oxidase system. Total protein concentration in liver homogenates was determined using the Coomassie blue method with bovine serum albumin as a standard.

Detection of hydroxyproline content in liver

Total collagen content in fresh liver samples was determined by hydroxyproline assay. Hydroxyproline content was detected with a commercial hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) following its manufacturer's instructions.

Measurement of serum HA, LN, and PⅢNP levels

Serum HA, LN and PⅢNP levels were measured by radioimmunoassay with a commercial kit according to its manufacturer's instructions (Beijing North Institute of Biological Technology, Beijing, China).

Statistical analysis

Data were analyzed with SPSS software. Quantitative data were presented as mean ± SD and analyzed by one way ANOVA analysis. Frequency data (pathologic grading of hepatic fibrosis) were analyzed by Ridit analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Pathological changes

Degeneration, necrosis, infiltration of inflammatory cells, and collagen deposition were found in liver tissues of model control group, CCl_4 + NAC group and 3 melatonin treatment groups (Figure 1C-F). Liver tissue samples from rats in normal control group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A and B). Formation of fibrotic septa encompassing regenerated hepatocytes was observed in liver tissue samples from rats in model group (Figure 1D). A large number of inflammatory cells infiltrated intra- and interlobular regions (Figure 1C). Statistical analysis revealed significant differences in pathologic grading between CCl_4 + MEL3 group and model control group ($P < 0.05$), indicating that fibrogenesis was much less severe in CCl_4 + MEL3 group than in model control group (Table 1, Figure 1C-F).

Table 1 Pathologic grading of hepatic fibrosis in different groups

Group	Dose (mg/kg)	*n*	Pathologic grading of hepatic fibrosis					*u* value
			0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	
Normal	-	11	11	0	0	0	0	5.5681[b]
Model	-	13	0	0	1	6	6	-
NAC	100	12	0	2	5	3	2	1.8838
MEL	2.5	11	0	2	4	2	3	1.5568
	5	13	0	2	4	3	4	1.3662
	10	12	0	4	4	1	3	2.1720[a]

u represents the Ridit value of the two groups, $P < 0.05$ indicates $u > 1.96$; $P < 0.01$ indicates $u > 2.58$; [a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

Table 2 Effect of melatonin on serum ALT, AST levels and A/G value in different groups (mean ± SD)

Group	Dose (mg/kg)	*n*	ALT (U/L)	AST (U/L)	A/G
Normal	-	11	70.00 ± 35.27	139.82 ± 72.83	0.94 ± 0.40
Model	-	13	286.23 ± 121.91[b]	431.00 ± 166.35[b]	0.74 ± 0.09
NAC	100	12	194.42 ± 90.83[b,c]	293.33 ± 94.60[b,c]	0.78 ± 0.11
MEL	2.5	11	211.09 ± 97.03[b]	357.09 ± 153.26[b]	0.68 ± 0.15
	5	13	201.15 ± 101.16[b,c]	321.23 ± 162.48[b,c]	0.77 ± 0.15
	10	12	178.67 ± 103.14[b,d]	292.42 ± 126.23[b,c]	0.73 ± 0.07

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; A: Albumin; G: Globumin. [b]$P < 0.01$ *vs* normal control group; [c]$P < 0.05$ *vs* model control group; [d]$P < 0.01$ *vs* model control group.

Detection of liver function

The serum ALT and AST levels were significantly higher in experimental and model groups than in normal control group ($P < 0.01$). The ALT and AST levels were significantly higher in model group than in CCl_4 + NAC and CCl_4 + MEL groups (5, 10 mg/kg) ($P < 0.05$, $P < 0.01$). Melatonin (5, 10 mg/kg) significantly decreased the elevated serum transaminase levels (Table 2), whereas no significant difference in the ratio of A/G was observed between model control group, CCl_4 + NAC and CCl_4 + MEL groups (Table 2).

MDA content and GPx activity in liver homogenates

The MDA level was significantly higher while GPx activity was significantly lower in liver homogenates of CCl_4 + NAC and CCl_4 + MEL groups than in those of normal control group ($P < 0.01$). The MDA level was significantly higher in model control group than in CCl_4 + NAC and CCl_4 + MEL groups (5, 10 mg/kg) ($P < 0.05$). Melatonin (5, 10 mg/kg) significantly blocked the elevated MDA level. GPx activity was significantly lower in the model control group than in CCl_4 + NAC and CCl_4 + MEL groups (5, 10 mg/kg) ($P < 0.05$, $P < 0.01$, Table 3).

HYP contents in liver tissue

Hepatic fibrosis was quantified by measuring hepatic

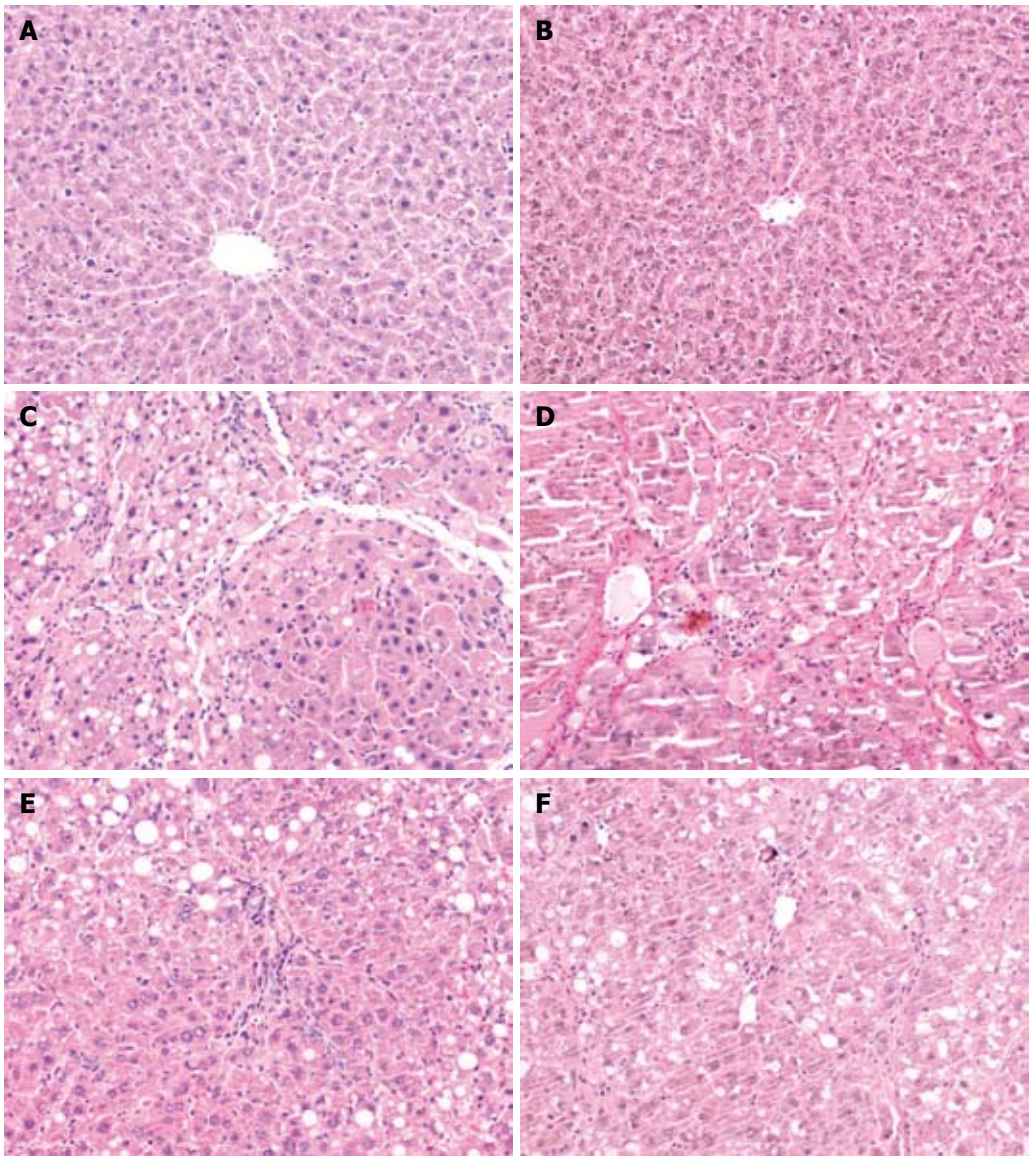

Figure 1 Pathological changes. Light microscopy of liver tissue sections showing normal liver lobular architecture with central veins in the normal control group (HE staining, × 200) (A), no collagen deposition in liver of normal control group (VG staining, × 200) (B), degenerated and necrotic liver cells associated with inflammatory cells in model group (HE staining, × 200) (C), formation of fibrotic septa in model group (VG staining, × 200) (D), and pathological change in liver of CCl_4 + melatonin (10 mg/kg) group was rather milder compared with the model group (HE staining, × 200; VG staining, × 200) (E, F).

Table 3 MDA content and GPx activity in liver homogenates of different groups (mean ± SD)

Group	Dose (mg/kg)	*n*	MDA (noml/mg prot)	GPx (U/mg prot)
Normal	-	11	4.3 ± 1.87	80.68 ± 10.76
Model	-	13	7.89 ± 1.49[b]	49.13 ± 8.72[b]
NAC	100	12	6.29 ± 1.36[b,c]	64.68 ± 8.22[b,d]
MEL	2.5	11	6.84 ± 1.10[b]	53.44 ± 9.35[b]
	5	13	6.29 ± 1.42[b,c]	57.38 ± 7.65[b,c]
	10	12	6.25 ± 2.27[b,c]	61.39 ± 13.15[b,d]

MDA: Malondialdehyde; GPx: Glutathione peroxidase. [b]$P < 0.01$ *vs* normal control group; [c]$P < 0.05$ *vs* model control group; [d]$P < 0.01$ *vs* model control group.

hydroxyproline. The hydroxyproline content was significantly higher in model control, CCl_4 + NAC and CCl_4 + MEL groups than in normal control group ($P < 0.01$), and significantly higher in model group than in CCl_4 + NAC and CCl_4 + MEL groups (10 mg/kg) ($P < 0.05$). Treatment with melatonin (10 mg/kg) or NAC reduced the hydroxyproline content in liver homogenates, and therefore prevented hepatic fibrosis induced by CCL_4 (Figure 2).

Measurement of serum HA, LN, and PⅢNP levels

The serum LN and HA levels were significantly higher in model control, CCl_4 + NAC, and CCl_4 + MEL groups than in normal control group ($P < 0.05$, $P < 0.01$), and significantly decreased after treatment with melatonin (10 mg/kg) ($P < 0.05$). Treatment with NAC significantly reduced the serum HA level ($P < 0.05$). The serum PⅢNP level was significantly higher in model control, CCl_4 + NAC and CCl_4 + MEL groups than in normal control group ($P < 0.05$). However, no significant difference was observed among the five groups (Table 4).

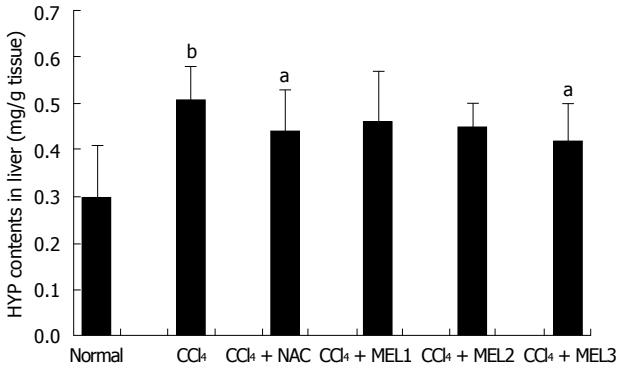

Figure 2 Effect of melatonin on hydroxyproline content in liver of rats with fibrosis fibrotic induced by CCl_4. $^aP < 0.05$ *vs* model control group; $^bP < 0.01$ *vs* normal control group.

Table 4 Serum HA, LN and PⅢNP levels in different groups (mean ± SD)

Group	Dose (mg/kg)	*n*	HA (μg/L)	LN (μg/L)	PⅢNP (μg/L)
Normal	-	11	71.65 ± 27.64	37.65 ± 6.09	26.41 ± 7.28
Model	-	13	201.10 ± 68.46^b	55.26 ± 12.30^b	35.88 ± 5.92^b
NAC	100	12	131.31 ± 58.58^{a,c}	48.28 ± 4.93^b	32.89 ± 3.90^b
MEL	2.5	11	174.41 ± 72.99^b	48.15 ± 6.40^b	34.21 ± 5.76^b
	5	13	153.54 ± 86.19^b	48.22 ± 9.51^b	33.12 ± 4.71^b
	10	12	135.71 ± 76.03^{a,c}	45.89 ± 11.71^{a,c}	31.99 ± 6.09^a

HA: Hyaluronic acid; LN: Laminin; PⅢNP: Procollagen Ⅲ N-terminal peptide. $^aP < 0.05$ *vs* normal control group; $^bP < 0.01$ *vs* normal control group; $^cP < 0.05$ *vs* model control group.

DISCUSSION

CCl_4 is widely used to induce hepatic fibrosis and cirrhosis in animal models. In this study, hepatic fibrosis was successfully induced by subcutaneous injection of sterile CCl_4 twice weekly for 12 wk. Through this hepatic fibrosis model, the effects of melatonin on hepatic fibrosis induced by CCl_4 in rats were examined.

N-acetylcysteine (NAC), a free radical scavenger, is a glutathione precursor which increases glutathione levels in hepatocytes[15,16]. Increased glutathione levels limit the production of reactive oxygen species (ROS) which can cause hepatocellular injury. NAC can also inhibit the proliferation of hepatic stellate cells[17]. Therefore, it was used as a positive control in this study.

It is well known that oxidative damage can induce hepatic fibrogenesis. ROS, such as H_2O_2, O_2^-, and $^{\cdot}OH$, are implicated in the development and pathological progress of hepatic fibrosis[18,19]. Free radicals and biomolecular reaction products promote phagocytic and myofibroblastic activities. Lipid peroxidation accelerates collagen synthesis by stimulating stellate cells[20]. It has been shown that melatonin is an effective antioxidant and a free radical scavenger. Due to its small size and high lipophilicity, melatonin can cross biological membranes easily and reach all compartments within the cell[21], thus protecting DNA, proteins, and biological membrane lipids from the deleterious effects of free radicals[22]. It has been found that melatonin has a higher antioxidant efficiency than vitamin E and GSH, which are known as powerful antioxidants[10]. The antioxidant properties of melatonin prevent acute liver injury induced by ischemia-reperfusion[23], irradiation[24], bile duct ligation[25-27], and toxins[18,28,29]. Several lines of evidence suggest that melatonin plays an important role in regulation of collagen levels and inhibition of collagen accumulation. Ostrowska *et al*[30] showed that melatonin is negatively related with urine hydroxyproline levels in fasting rats. Cunnane *et al*[31] demonstrated that primary biliary cirrhosis is related with melatonin deficiency in pinealectomized rats. Tahan *et al*[12] reported that daily melatonin injection at pharmacological doses is effective against liver damage in a rat liver fibrosis model induced by a 14-d dimethylnitrosamine regimen. In the present study, liver injury was assessed with histological and biochemical parameters. The results suggest that melatonin could decrease the scores of hepatic fibrosis and serum ALT and AST levels in rats with hepatic injury caused by CCl_4. Melatonin at a dose of 10 mg/kg was as effective as 100 mg/kg NAC in reducing serum ALT and AST levels, indicating that melatonin can protect liver and alleviate the progression of hepatic fibrosis. However, further study is needed on the liver function protective effect of melatonin in cirrhotic patients.

HA and LN are known to be good serum markers of hepatic fibrogenesis[32-34]. HYP in liver is an important index reflecting the degree of hepatic fibrosis and hepatic fibrosis can be quantified by measuring hepatic hydroxyproline[33,35]. In the present study, treatment with melatonin (10 mg/kg) could significantly reduce HA and LN in serum and HYP in liver. The decreased of hepatic hydroxyproline and serum LN and HA levels indicate that melatonin can inhibit collagen deposition in liver.

Oxidative stress plays an important role in the formation of hepatic fibrosis *via* increasing stellate cell activation and collagen synthesis. MDA is the main product of lipid peroxidation and its concentration is generally presented as the total level of lipid peroxidation products[36]. As an end product of lipid peroxidation, MDA can produce ozone, which reacts rapidly with cellular structures, generates hydrogen peroxide and other reactive oxygen species, leading to peroxidation and denaturation of membranes[37]. It has been shown that MDA can activate stellate cells that produce collagen. The results of this study suggest that treatment with melatonin (5, 10 mg/kg) could significantly block increased MDA, suggesting that melatonin decreases lipid peroxidation and plays an anti-oxidative role in hepatic fibrosis induced by CCl_4 in rats.

Melatonin is not only a direct antioxidant but also an indirect antioxidant through enhancement of antioxidant enzyme activities in liver[38]. It was reported that melatonin can reduce free radical damage by elevating GPx activation[11,39]. Montilla *et al*[25] reported that acute ligation of the bile duct is accompanied with decreased GSH levels both in plasma and in liver, as well as significantly reduced antioxidant enzyme activities. Treatment with melatonin is associated with a significant recovery of anti-oxidative enzymes such as

GPx[25]. Tahan *et al*[12] found that melatonin can restore GPx activity in a rat liver fibrosis model induced by a 14-d dimethylnitrosamine regimen. In this study, the GPx activity was significantly lower in model control group than in CCl_4 + NAC and CCl_4 + MEL groups (5, 10 mg/kg), indicating that melatonin can protect liver against CCl_4-induced hepatic fibrosis in rats, possibly through its direct and indirect antioxidant effects.

In conclusion, melatonin may have beneficial effects on hepatic fibrosis induced by CCl_4 in rats. The protective effect of melatonin on hepatic fibrosis may be related to its antioxidant activities.

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COMMENTS

Background

In China, the incidence of liver cirrhosis is still high. Liver cirrhosis results from fibrosis. If treated properly at fibrosis stage, cirrhosis can be prevented. However, no effective antifibrosis drugs are available at present. Several lines of evidences suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis. Melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free-radical-generating agents and processes. The possible fibrosuppressant effect of melatonin on hepatic fibrosis was evaluated in this study. In addition, the antioxidant and anti-inflammatory properties of melatonin were investigated in rats with fibrosis.

Research frontiers

Although the exact pathogenesis of hepatic fibrosis is still obscure, the role of free radicals and lipid peroxides in the development of hepatic fibrosis has attracted considerable attention. If treated properly at this stage, cirrhosis can be successfully prevented. However, it remains a problem to prevent hepatic fibrosis or to control its progression. Great efforts have been made to find safe and effective drugs. Recent experiments demonstrate that melatonin may have therapeutic effects on acute and chronic liver injury, possibly through its antioxidant activities.

Innovations and breakthroughs

Melatonin may have beneficial effects on hepatic fibrosis induced by carbon tetrachloride in rats. The protective effect of melatonin may be related to its antioxidant activities.

Applications

Melatonin can be used as an antifibrotic drug, protect liver cells against fibrosis and inhibits collagen fiber deposition in liver, thus providing a basis for further studies on its therapeutic effect on hepatic fibrosis.

Terminology

Melatonin (N-acetyl-5-metyoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant. It regulates circadian rhythms, sleep and immune system activity, behaves as a free radical scavenger, and eliminates oxygen free radicals and reactive intermediates.

Peer review

This is a well-designed study describing the protective effect of melatonin on fibrosis induced by carbon tetrachloride. Methods are appropriate and results are consistent with the conclusion. The study is very interesting with a great amount of data, which corroborate the major conclusion.

REFERENCES

1 **Pinzani M**, Rombouts K. Liver fibrosis: from the bench to clinical targets. *Dig Liver Dis* 2004; **36**: 231-242
2 **Parola M**, Robino G. Oxidative stress-related molecules and liver fibrosis. *J Hepatol* 2001; **35**: 297-306
3 **Shimizu I**. Antifibrogenic therapies in chronic HCV infection. *Curr Drug Targets Infect Disord* 2001; **1**: 227-240
4 **Allegra M**, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA. The chemistry of melatonin's interaction with reactive species. *J Pineal Res* 2003; **34**: 1-10
5 **Tan DX**, Manchester LC, Reiter RJ, Plummer BF, Hardies LJ, Weintraub ST, Vijayalaxmi, Shepherd AM. A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of in vivo hydroxyl radical generation. *Biochem Biophys Res Commun* 1998; **253**: 614-620
6 **Tan DX**, Manchester LC, Reiter RJ, Plummer BF, Limson J, Weintraub ST, Qi W. Melatonin directly scavenges hydrogen peroxide: a potentially new metabolic pathway of melatonin biotransformation. *Free Radic Biol Med* 2000; **29**: 1177-1185
7 **Longoni B**, Salgo MG, Pryor WA, Marchiafava PL. Effects of melatonin on lipid peroxidation induced by oxygen radicals. *Life Sci* 1998; **62**: 853-859
8 **Stasica P**, Ulanski P, Rosiak JM. Melatonin as a hydroxyl radical scavenger. *J Pineal Res* 1998; **25**: 65-66
9 **Zang LY**, Cosma G, Gardner H, Vallyathan V. Scavenging of reactive oxygen species by melatonin. *Biochim Biophys Acta* 1998; **1425**: 469-477
10 **Rozov SV**, Filatova EV, Orlov AA, Volkova AV, Zhloba AR, Blashko EL, Pozdeyev NV. N1-acetyl-N2-formyl-5-methoxykynuramine is a product of melatonin oxidation in rats. *J Pineal Res* 2003; **35**: 245-250
11 **Sener-Muratoğlu G**, Paskaloğlu K, Arbak S, Hürdağ C, Ayanoğlu-Dülger G. Protective effect of famotidine, omeprazole, and melatonin against acetylsalicylic acid-induced gastric damage in rats. *Dig Dis Sci* 2001; **46**: 318-330
12 **Tahan V**, Ozaras R, Canbakan B, Uzun H, Aydin S, Yildirim B, Aytekin H, Ozbay G, Mert A, Senturk H. Melatonin reduces dimethylnitrosamine-induced liver fibrosis in rats. *J Pineal Res* 2004; **37**: 78-84
13 **Cruz A**, Padillo FJ, Torres E, Navarrete CM, Muñoz-Castañeda JR, Caballero FJ, Briceño J, Marchal T, Túnez I, Montilla P, Pera C, Muntané J. Melatonin prevents experimental liver cirrhosis induced by thioacetamide in rats. *J Pineal Res* 2005; **39**: 143-150
14 **Scheuer PJ**. Classification of chronic viral hepatitis: a need for reassessment. *J Hepatol* 1991; **13**: 372-374
15 **Doğru-Abbasoğlu S**, Balkan J, Kanbağli O, Cevikbaş U, Aykaç-Toker G, Uysal M. Aminoguanidine, an inducible nitric oxide synthase inhibitor, plus N-acetylcysteine treatment reduce the lipopolysaccharide-augmented hepatotoxicity in rats with cirrhosis. *Hum Exp Toxicol* 2002; **21**: 359-364
16 **Vendemiale G**, Grattagliano I, Caruso ML, Serviddio G, Valentini AM, Pirrelli M, Altomare E. Increased oxidative stress in dimethylnitrosamine-induced liver fibrosis in the rat: effect of N-acetylcysteine and interferon-alpha. *Toxicol Appl Pharmacol* 2001; **175**: 130-139
17 **Kim KY**, Rhim T, Choi I, Kim SS. N-acetylcysteine induces cell cycle arrest in hepatic stellate cells through its reducing activity. *J Biol Chem* 2001; **276**: 40591-40598
18 **Serviddio G**, Pereda J, Pallardó FV, Carretero J, Borras C, Cutrin J, Vendemiale G, Poli G, Viña J, Sastre J. Ursodeoxycholic acid protects against secondary biliary cirrhosis in rats by preventing mitochondrial oxidative stress. *Hepatology* 2004; **39**: 711-720
19 **Lu G**, Shimizu I, Cui X, Itonaga M, Tamaki K, Fukuno H, Inoue H, Honda H, Ito S. Antioxidant and antiapoptotic activities of idoxifene and estradiol in hepatic fibrosis in rats. *Life Sci* 2004; **74**: 897-907
20 **Geesin JC**, Hendricks LJ, Falkenstein PA, Gordon JS, Berg RA. Regulation of collagen synthesis by ascorbic acid: characterization of the role of ascorbate-stimulated lipid peroxidation. *Arch Biochem Biophys* 1991; **290**: 127-132
21 **Sener G**, Jahovic N, Tosun O, Atasoy BM, Yeğen BC. Melatonin ameliorates ionizing radiation-induced oxidative organ damage in rats. *Life Sci* 2003; **74**: 563-572
22 **Reiter RJ**, Poeggeler B, Tan DX, Chen LD, Manchester LC, Guerrero JM. Antioxidant capacity of melatonin: A novel

action not requiring a receptor. *Neuroendocrinol Lett* 1993; **15**: 103-116
23 **Zavodnik LB**, Zavodnik IB, Lapshina EA, Belonovskaya EB, Martinchik DI, Kravchuk RI, Bryszewska M, Reiter RJ. Protective effects of melatonin against carbon tetrachloride hepatotoxicity in rats. *Cell Biochem Funct* 2005; **23**: 353-359
24 **El-Missiry MA**, Fayed TA, El-Sawy MR, El-Sayed AA. Ameliorative effect of melatonin against gamma-irradiation-induced oxidative stress and tissue injury. *Ecotoxicol Environ Saf* 2007; **66**: 278-286
25 **Montilla P**, Cruz A, Padillo FJ, Túnez I, Gascon F, Muñoz MC, Gómez M, Pera C. Melatonin versus vitamin E as protective treatment against oxidative stress after extra-hepatic bile duct ligation in rats. *J Pineal Res* 2001; **31**: 138-144
26 **Ohta Y**, Kongo M, Kishikawa T. Melatonin exerts a therapeutic effect on cholestatic liver injury in rats with bile duct ligation. *J Pineal Res* 2003; **34**: 119-126
27 **Padillo FJ**, Cruz A, Navarrete C, Bujalance I, Briceño J, Gallardo JI, Marchal T, Caballero R, Túnez I, Muntané J, Montilla P, Pera-Madrazo C. Melatonin prevents oxidative stress and hepatocyte cell death induced by experimental cholestasis. *Free Radic Res* 2004; **38**: 697-704
28 **El-Sokkary GH**, Abdel-Rahman GH, Kamel ES. Melatonin protects against lead-induced hepatic and renal toxicity in male rats. *Toxicology* 2005; **213**: 25-33
29 **Sigala F**, Theocharis S, Sigalas K, Markantonis-Kyroudis S, Papalabros E, Triantafyllou A, Kostopanagiotou G, Andreadou I. Therapeutic value of melatonin in an experimental model of liver injury and regeneration. *J Pineal Res* 2006; **40**: 270-279
30 **Ostrowska Z**, Swientochowska E, Buntner B, Marek B, Zwirska-Korczala K, Spyra Z, Banas I I. Assessment of relationship between secretion of melatonin, activity of thyroid, adrenal cortex as well as testes and chosen markers of collagen metabolism in starved rats. *Endocr Regul* 1996; **30**: 83-92
31 **Cunnane SC**, Manku MS, Horrobin DF. The pineal and regulation of fibrosis: pinealectomy as a model of primary biliary cirrhosis: roles of melatonin and prostaglandins in fibrosis and regulation of T lymphocytes. *Med Hypotheses* 1979; **5**: 403-414
32 **Kaneda H**, Hashimoto E, Yatsuji S, Tokushige K, Shiratori K. Hyaluronic acid levels can predict severe fibrosis and platelet counts can predict cirrhosis in patients with nonalcoholic fatty liver disease. *J Gastroenterol Hepatol* 2006; **21**: 1459-1465
33 **Dang SS**, Wang BF, Cheng YA, Song P, Liu ZG, Li ZF. Inhibitory effects of saikosaponin-d on CCl4-induced hepatic fibrogenesis in rats. *World J Gastroenterol* 2007; **13**: 557-563
34 **Li CH**, Pan LH, Yang ZW, Li CY, Xu WX. Preventive effect of Qianggan-Rongxian Decoction on rat liver fibrosis. *World J Gastroenterol* 2008; **14**: 3569-3573
35 **Hayasaka A**, Saisho H. Serum markers as tools to monitor liver fibrosis. *Digestion* 1998; **59**: 381-384
36 **Drewa G**, Krzyzyńska-Malinowska E, Woźniak A, Protas-Drozd F, Mila-Kierzenkowska C, Rozwodowska M, Kowaliszyn B, Czajkowski R. Activity of superoxide dismutase and catalase and the level of lipid peroxidation products reactive with TBA in patients with psoriasis. *Med Sci Monit* 2002; **8**: BR338-BR343
37 **Ajamieh HH**, Menéndez S, Martínez-Sánchez G, Candelario-Jalil E, Re L, Giuliani A, Fernández OS. Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischaemia-reperfusion. *Liver Int* 2004; **24**: 55-62
38 **Liu F**, Ng TB. Effect of pineal indoles on activities of the antioxidant defense enzymes superoxide dismutase, catalase, and glutathione reductase, and levels of reduced and oxidized glutathione in rat tissues. *Biochem Cell Biol* 2000; **78**: 447-453
39 **Wang H**, Xu DX, Lv JW, Ning H, Wei W. Melatonin attenuates lipopolysaccharide (LPS)-induced apoptotic liver damage in D-galactosamine-sensitized mice. *Toxicology* 2007; **237**: 49-57

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Risk of contrast-induced nephropathy in hospitalized patients with cirrhosis

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Abstract

AIM: To evaluate the incidence of contrast-induced nephropathy (CIN) in cirrhotic patients and to identify risk factors for the development of CIN.

METHODS: We performed a retrospective review of 216 consecutive patients with cirrhosis who underwent computed tomography (CT) with intravenous contrast at the University of Rochester between the years 2000-2005. We retrospectively examined factors associated with a high risk for CIN, defined as a decrease in creatinine clearance of 25% or greater within one week after receiving contrast.

RESULTS: Twenty-five percent of our patients developed CIN, and 74% of these patients had ascites seen on CT. Of the 75% of patients who did not develop CIN, only 46% had ascites. The presence of ascites was a significant risk factor for the development of CIN (P = 0.0009, OR 3.38, 95% CI 1.55-7.34) in multivariate analysis. Patient age, serum sodium, Model for End-stage Liver Disease score, diuretic use, and the presence of diabetes were not found to be significant risk factors for the development of CIN. Of the patients who developed CIN, 11% developed chronic renal insufficiency, defined as a creatinine clearance less than baseline for 6 wk.

CONCLUSION: Our results suggest that in hospitalized cirrhotic patients, especially those with ascites, the risk of CIN is substantial.

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Key words: Ascites; Cirrhosis; Contrast-induced nephropathy; Renal failure

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INTRODUCTION

Renal failure is associated with a high morbidity and mortality in patients with cirrhosis[1-4]. Cirrhotic patients may be particularly predisposed to renal failure because of intravascular volume depletion, hyperaldosteronism, and altered renal hemodynamics[5]. Furthermore, aggressive use of diuretics, repeated large volume paracenteses, and gastrointestinal bleeding often contribute to renal insufficiency in these patients[1,2].

Contrast-induced nephropathy (CIN) is a common cause of renal failure, and is associated with substantial morbidity and mortality[6-9]. Multiple studies in the medical literature have estimated a risk of 2% in low-risk patients, rising to 50% in those with risk factors such as diabetes mellitus (DM), pre-existing renal disease, congestive heart failure, advanced age, anemia, and dehydration[10-15].

Although cirrhosis has been suggested as a risk factor for CIN[13-17], only two studies, to our knowledge, have specifically investigated this issue. A prospective study by Guevara *et al*[18] did not find an increased susceptibility to CIN in cirrhotic patients. Although this was a well-conducted study, it was conducted under idealized circumstances. Our goal was to conduct a study that may be more representative of the cirrhotic patients we encounter on a frequent basis[18]. The study by Najjar

et al[19] was a retrospective study which also did not find an increased prevalence of CIN in cirrhotics. However, the authors did not precisely define CIN, making it difficult to interpret the results of the study[15]. We believe that cirrhosis is a risk factor for the development of CIN, and we aimed to identify risk factors for the development of CIN. We thus performed a retrospective review of cirrhotics who received iodinated contrast during hospitalization for further analysis.

MATERIALS AND METHODS

After approval by the University of Rochester's Institutional Review Board, we reviewed the charts of 347 patients with cirrhosis who underwent computed tomography (CT) with intravenous contrast during hospitalization at our institution between the years 2000-2005. Inclusion criteria consisted of the presence of cirrhosis, inpatient hospitalization, and the use of iodinated contrast for CT. Patients with pre-existing sepsis, known congestive heart failure, gastrointestinal bleeding, spontaneous bacterial peritonitis (SBP), and chronic kidney disease (CKD), defined as baseline Cr > 18 mg/L, were excluded from our study. Patients who received peri-contrast intravenous sodium chloride, sodium bicarbonate, N-acetylcysteine, as well as those whose diuretic therapy was held during the day of contrast exposure were also excluded from our study.

Clinical data that were reviewed included the Model for End-stage Liver Disease (MELD) score, use of diuretics, serum sodium, a documented diagnosis of diabetes mellitus (DM) in the medical record, and the presence of ascites seen on CT. The use of potentially nephrotoxic medications, such as angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin receptor blockers (ARBs), nonsteroidal anti-inflammatory drugs (NSAIDs), and aspirin was documented. The age and race of each patient were also noted. Statistical analysis was performed by faculty of the Department of Biostatistics at the University of Rochester. Multivariate and univariate analyses were performed using logistic regression, and odds ratios were calculated.

All patients received 150 mL of iodinated contrast dye (iohexol, Omnipaque) intravenously per standard radiology protocol. Post-contrast creatinine was then reviewed and compared to baseline values. Serum creatinine on the day of contrast exposure was used to calculate the pre-contrast creatinine clearance (CrCl). Post-contrast CrCl was determined by the highest recorded creatinine value within one week after contrast exposure. The aim of this study was to evaluate the development of CIN, which we defined as a decrease in CrCl of 25% or greater, temporally associated with the use of contrast. CrCl was estimated using the Cockroft-Gault equation. Patients who developed CIN were followed for 6 wk to assess the development of chronic renal insufficiency (CRI), defined as a CrCl less than baseline for 6 wk. The need for dialysis during this time period was also documented.

Table 1 Demographic data of the 216 patients included in the study

Factor	Patients	Percentage
Mean age	53.2	N/A
> 65 YO	34	16
< 65 YO	182	84
Male	128	59
Female	88	41
Caucasian	158	73
African American	37	17
Hispanic	10	4
Asian	3	1
Unknown	8	5

Table 2 Characteristics of patients who developed CIN (*n* = 53) *vs* those who did not develop CIN (*n* = 163)

Factor	Percent with CIN	Percent without CIN	*P*-value
Ascites	74	46	0.0009
MELD > 15	60	48	0.1774
Age > 65	9	18	0.2171
On diuretics	66	57	0.3200
Na ⩾ 130	91	88	0.8449

Table 3 Relationship of ascites to incidence of CIN

Factor	Patients	Percentage
Ascites	114	53
CIN	39	34
No CIN	75	66
No ascites	102	47
CIN	14	14
No CIN	88	86

Ascites was a significant risk factor for the development of CIN ($P = 0.0009$, OR 3.38, CI 1.55-7.34).

RESULTS

A total of 216 patients met the inclusion criteria. 34 patients were greater than 65 years of age (16%). The mean age was 53.2 years. 158 patients were Caucasian, 37 were African American, 10 were Hispanic, and 3 were Asian (Table 1). The mean MELD score for all patients was 15.3.

A total of 53 (25%) patients developed CIN. Baseline characteristics of these patients included a mean MELD of 17, mean age of 51.9, and mean serum sodium of 134.7 mmol/L. In 36 of these patients (68%), renal insufficiency persisted for at least one week. A total of 39 (74%) of these patients had ascites (Table 2). Although 6 patients (11%) developed CRI, none of those patients required dialysis during our 6 wk review.

A total of 163 patients (75%) did not develop CIN. Their mean MELD score was 14.8, mean age was 53.6, and mean sodium was 135.8 mmol/L. Seventy-five of these patients (46%) had ascites seen on CT (Table 2).

The presence of ascites was a significant risk factor for the development of CIN ($P = 0.0009$, OR 3.38, 95% CI 1.55-7.34) in multivariate analyses (Table 3, Figure 1).

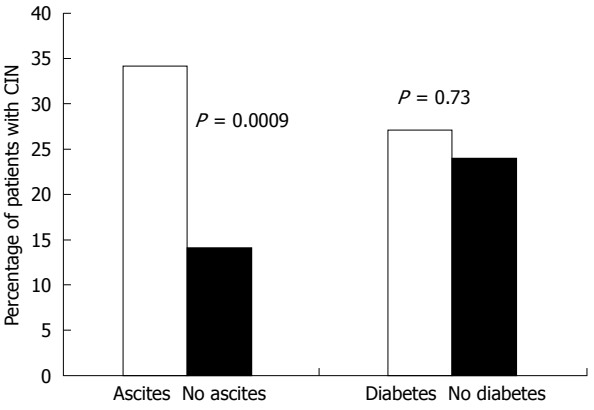

Figure 1 Percentage of patients experiencing CIN in the presence or absence of ascites (*P* = 0.0009) or diabetes (*P* = 0.73).

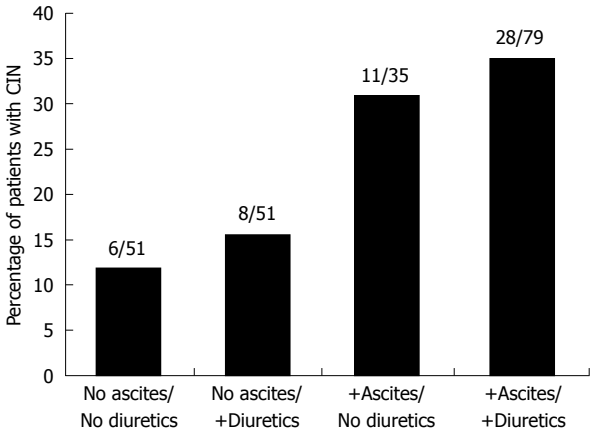

Figure 2 Percentage of patients experiencing CIN in relation to diuretic use and the presence of ascites.

A total of 33 patients were on ACE inhibitors, ARBs, NSAIDs, or aspirin. Ascites remained a significant risk factor when these patients were excluded from this analysis (*P* = 0.00006, OR 3.98, 95% CI 1.83-8.69). Ascites was also a significant risk factor for the development of CRI, as 5/6 patients (83%) who developed CRI had ascites seen on CT scan. Age, serum sodium, and MELD score were not found to be significantly associated with a higher risk of CIN in multivariate analysis. Our analysis also did not show a significant association between the use of diuretics and an increased risk of CIN in patients with or without ascites (Figure 2).

In our study, the presence of DM was not a predisposing factor to CIN (Figure 1). A total of 66 diabetic patients were included in the analysis. The incidence of CIN was 18/66 (27%) in these patients, a nonsignificant difference compared to nondiabetics, where the incidence of CIN was 36/150 (24%). Among the total number of diabetic patients, three had evidence of mild kidney disease prior to the scan, defined as Cr > 15 mg/L. Only one (33%) of these patients developed CIN.

DISCUSSION

Intravenous contrast remains an important cause of acute renal failure in patients who receive CT scans. There is little data on whether the presence of cirrhosis serves as an important risk factor for the development of CIN.

We performed a large retrospective review at our institution of hospitalized cirrhotic patients who received intravenous contrast for CT imaging and found that there was a high rate of CIN. In multivariate analysis, the presence of ascites was a significant risk factor for the development of CIN, conferring over three times the risk compared to the absence of ascites. Factors such as MELD score, serum sodium, diuretic use, the presence of DM, and age failed to show a similar association.

The results of this study are dissimilar to those found in a prospective study by Guevara *et al*[18], who did not find an increased susceptibility to CIN in cirrhotic patients[18]. However, this study was limited by a relatively small sample size and diuretic therapy was withheld for at least 5 days prior to inclusion in the study. Our study included patients on diuretic therapy, and is therefore reflective of a broad range of cirrhotic patients.

A second retrospective study by Najjar *et al*[19] comparing 72 cirrhotic patients receiving intravenous contrast with 72 non-cirrhotic controls, revealed the development of CIN in 2 patients with cirrhosis (2.8%) and in 1 patient in the control group (1.4%), a non-significant difference. The authors of this study concluded that cirrhosis may not be a risk factor for CIN. However, the results of this study should be interpreted with caution because the study does not clearly define CIN[19]. Without a precise definition of CIN, it is difficult to interpret the results of this study.

The exact mechanism by which iodinated contrast agents induce renal dysfunction is not entirely understood. The pathophysiology is complex, and a variety of factors act in concert to induce CIN. *In vitro* and animal studies suggest that damage secondary to iodinated contrast to the kidneys is likely mediated by a combination of toxic or obstructive injury to the renal tubules, ischemic injury by reactive oxygen species, and renal medullary hypoxia. The predominant factor is likely to be renal medullary hypoxia, in which adenosine, calcium, and endothelin bring about intrarenal vasoconstriction after contrast exposure[17,20-25]. Tubular toxicity is also thought to play a role in CIN through both direct nephrotoxicity and tubular obstruction. The generation of reactive oxygen species can cause toxic, ischemic, and immune-mediated direct nephrotoxicity[26-28]. Contrast dye increases urate excretion and leads to the deposition of Tamm-Horsfall proteins within the renal tubules, both of which can cause tubular obstruction[29-31].

Factors such as age, serum sodium, MELD score, and diuretic use were not found to be associated with the development of CIN. It is surprising that there is no statistically significant relationship between diuretic use and CIN in our study. There are a fair number of studies suggesting that peri-contrast hydration can reduce the incidence of CIN[32-36]. Furthermore, prophylactic forced diuresis with furosemide has been shown to

augment the risks of CIN[32]. However, there are no data, to our knowledge, on the association between maintenance diuretic therapy and CIN. Although an increased frequency of CIN in the setting of diuretic use is biologically plausible, this relationship should be explored further before any conclusions can be made.

In our analysis, the presence of DM did not confer an increased risk of CIN. Although DM is considered to be a risk factor for CIN, the data on whether this relationship is present independent of underlying renal impairment are conflicting[7,8,37]. Only three patients with DM in our analysis had evidence of mild kidney disease before receiving contrast, and one of these developed CIN. Because our study was retrospective, we were unable to assess our patients for the presence of microalbuminuria or overt proteinuria. Furthermore, our exclusion criteria of heart failure and CKD may have excluded many diabetic patients with significant vascular disease. Our sample may thus have consisted of a higher proportion of patients with uncomplicated diabetes and therefore at a lower risk of CIN.

In those patients that developed CIN, a large proportion (68%) had CIN that persisted for at least one week. 11% of these patients developed CRI as a possible result of contrast exposure. Although none of these patients required dialysis, even transient elevations in creatinine without progression to dialysis have been associated with prolonged hospital stay, adverse cardiac events, and increased mortality[6-9].

There have been multiple studies performed investigating whether certain prophylactic regimens may reduce the risk of CIN. These have included agents such as N-acetylcysteine, diuretics, dopamine, hemofiltration, as well as hydration with sodium chloride or sodium bicarbonate[38]. However, none of these have been performed in cirrhotic patients, and reviews of these trials have given discrepant results. Currently, only the use of low osmolality contrast medium at the lowest dose possible, in conjunction with saline hydration is recommended in a recent review article[17].

The primary limitation of our study is the use of serum creatinine in determining the incidence of CIN. The assessment of renal function is notoriously difficult in patients with cirrhosis, and creatinine is likely a sub-optimal measure of renal function in these patients. Although we assessed CrCl rather than an absolute change in serum creatinine, studies suggest that creatinine-based formulas (e.g. Cockroft-Gault, Modification of Diet in Renal Disease) can only provide a crude approximation of true glomerular filtration rate (GFR) in cirrhotics[38-40]. However, direct measurement of kidney function (e.g. inulin clearance) is impractical in large cohorts, and cystatin C-based equations (e.g. Larsson and Hoek) are also unable to accurately assess GFR in patients with cirrhosis[41]. Furthermore, the prognostic impact of serum creatinine in patients with cirrhosis is well-validated[5,42-45], and its predictive value is reflected by its inclusion in the MELD score. Additionally, numerous studies have used either a 25% rise in serum creatinine or a 25% decrease in estimated GFR to assess the development of CIN[7-9,11,12,34,38]. While not ideal, we feel that using a 25% decrease in CrCl is an adequate means of detecting clinically significant CIN.

Another important limitation of our study is that we defined CIN based solely upon the peak CrCl within one week after receiving contrast. Although patients with heart failure, SBP, sepsis, and CKD were excluded from the study, there are other causes of increases in serum creatinine in cirrhotic patients exposed to intravenous contrast (e.g. hepatorenal syndrome, large volume paracenteses). Fluctuations in serum creatinine are common in the inpatient setting, and often no discernable cause is found for these variations[46]. It is therefore possible that other factors may have contributed to alterations in CrCl in some of our patients. However, in hospitalized patients with no other apparent cause for a decline in renal function temporally associated with the administration of intravenous contrast, it is difficult to exclude CIN as a major contributing factor for this deterioration.

The retrospective nature of our study creates many limitations on our research, most notably the lack of a control group without intravenous contrast administration. The presence of a control group is particularly important when evaluating the incidence of CIN, as fluctuations in serum creatinine can have a multitude of causes. There are only two published studies, to our knowledge, that specifically compare the incidence of post-contrast renal dysfunction with the incidence of renal dysfunction in a control group of patients who did not receive intravenous contrast. Neither of these studies attributed a significant difference in the risk of renal failure to intravenous contrast, suggesting that the risk of CIN may be exaggerated. However, both of these studies were limited by a lack of randomization and a high threshold for the diagnosis of renal dysfunction (defined as a 50% increase in serum creatinine), and it is possible that differences in methodology may account for their findings[47,48]. Nonetheless, future studies assessing the risk of CIN in cirrhotic patients would be strengthened by the inclusion of a parallel control group[49,50].

It is also important to note that although diuretic use did not have an independent association with the development of CIN, there may be an association that our retrospective analysis was not able to elucidate. Patients with ascites are the most likely to receive high doses of diuretic therapy. It is difficult to sub-classify patients on the aggressiveness of their diuretic therapy, and we were thus unable to differentiate between patients on minimal doses of diuretics and those receiving aggressive diuresis. It is possible that high doses of diuretics may be a significant contributing factor to the development of CIN in patients with ascites. In patients with ascites receiving large doses of diuretic therapy, our retrospective study was unable to differentiate whether the development of CIN was from volume depletion from diuretic use or whether ascites was an independent predisposing factor.

In conclusion, our large retrospective study of hospitalized cirrhotic patients revealed a high incidence of CIN, especially in patients with ascites. CIN was associated with a significant percentage of patients progressing to CRI as a likely result of contrast exposure. These results suggest that in hospitalized cirrhotic patients, especially those with ascites, the risk of CIN is substantial. Alternative imaging strategies should be considered, and post-contrast renal function should be meticulously followed. Prospective studies evaluating the risk of contrast-induced nephropathy in cirrhotic patients, with and without the presence of ascites, should be performed for further investigation.

COMMENTS

Background

Contrast-Induced Nephropathy (CIN) is associated with substantial morbidity and mortality, especially in patients with cirrhosis. It is therefore important to determine whether cirrhosis is a risk factor for CIN, as well as to investigate which, if any, cirrhotic patients are particularly prone to CIN.

Research frontiers

The medical implications of CIN are substantial. As a result, there is a significant amount of research taking place on the identification of risk factors for CIN, as well as strategies to prevent its development. However, despite the vast amount of research on CIN, the data on the association between cirrhosis and CIN is scarce.

Applications

The study results suggest that in hospitalized cirrhotic patients, especially those with ascites, the risk of CIN is substantial.

Terminology

CIN: A decrease in CrCl of 25% or greater within one week after contrast exposure; Chronic Renal Insufficiency (CRI): a CrCl less than baseline for 6 wk; Chronic Kidney Disease (CKD): baseline Cr > 18 mg/L; Mild Kidney Disease: baseline Cr > 15 mg/L.

Peer review

Lodhia *et al* report the findings of a retrospective study that measured the risk of CIN in patients with cirrhosis. They found that the incidence of CIN was increased and that ascites was an independent risk factor for CIN. This is a very interesting and well written study.

REFERENCES

1 **Ginès A**, Escorsell A, Ginès P, Saló J, Jiménez W, Inglada L, Navasa M, Clària J, Rimola A, Arroyo V. Incidence, predictive factors, and prognosis of the hepatorenal syndrome in cirrhosis with ascites. *Gastroenterology* 1993; **105**: 229-236

2 **Wu CC**, Yeung LK, Tsai WS, Tseng CF, Chu P, Huang TY, Lin YF, Lu KC. Incidence and factors predictive of acute renal failure in patients with advanced liver cirrhosis. *Clin Nephrol* 2006; **65**: 28-33

3 **du Cheyron D**, Bouchet B, Parienti JJ, Ramakers M, Charbonneau P. The attributable mortality of acute renal failure in critically ill patients with liver cirrhosis. *Intensive Care Med* 2005; **31**: 1693-1699

4 **Alessandria C**, Ozdogan O, Guevara M, Restuccia T, Jiménez W, Arroyo V, Rodés J, Ginès P. MELD score and clinical type predict prognosis in hepatorenal syndrome: relevance to liver transplantation. *Hepatology* 2005; **41**: 1282-1289

5 **Epstien M**. Deranged renal function in liver disease. *Contrib Nephrol* 1977; **7**: 250-271

6 **Levy EM**, Viscoli CM, Horwitz RI. The effect of acute renal failure on mortality. A cohort analysis. *JAMA* 1996; **275**: 1489-1494

7 **Dangas G**, Iakovou I, Nikolsky E, Aymong ED, Mintz GS, Kipshidze NN, Lansky AJ, Moussa I, Stone GW, Moses JW, Leon MB, Mehran R. Contrast-induced nephropathy after percutaneous coronary interventions in relation to chronic kidney disease and hemodynamic variables. *Am J Cardiol* 2005; **95**: 13-19

8 **Mehran R**, Aymong ED, Nikolsky E, Lasic Z, Iakovou I, Fahy M, Mintz GS, Lansky AJ, Moses JW, Stone GW, Leon MB, Dangas G. A simple risk score for prediction of contrast-induced nephropathy after percutaneous coronary intervention: development and initial validation. *J Am Coll Cardiol* 2004; **44**: 1393-1399

9 **From AM**, Bartholmai BJ, Williams AW, Cha SS, McDonald FS. Mortality associated with nephropathy after radiographic contrast exposure. *Mayo Clin Proc* 2008; **83**: 1095-1100

10 **Mathew R**, Haque K, Woothipoom W. Acute renal failure induced by contrast medium: steps towards prevention. *BMJ* 2006; **333**: 539-540

11 **Manske CL**, Sprafka JM, Strony JT, Wang Y. Contrast nephropathy in azotemic diabetic patients undergoing coronary angiography. *Am J Med* 1990; **89**: 615-620

12 **Nikolsky E**, Mehran R, Lasic Z, Mintz GS, Lansky AJ, Na Y, Pocock S, Negoita M, Moussa I, Stone GW, Moses JW, Leon MB, Dangas G. Low hematocrit predicts contrast-induced nephropathy after percutaneous coronary interventions. *Kidney Int* 2005; **67**: 706-713

13 **Abraham PA**, Kjellstrand CM. Contrast media nephropathy. In: Massry SG, Glassock RJ. Textbook of nephrology. 2nd ed. Baltimore: Williams & Wilkins, 1989: 851-859

14 **Moreau R**, Lebrec D. Acute renal failure in patients with cirrhosis: perspectives in the age of MELD. *Hepatology* 2003; **37**: 233-243

15 **Barrett BJ**, Parfrey PS. Prevention of nephrotoxicity induced by radiocontrast agents. *N Engl J Med* 1994; **331**: 1449-1450

16 **Barrett BJ**. Contrast nephrotoxicity. *J Am Soc Nephrol* 1994; **5**: 125-137

17 **Barrett BJ**, Parfrey PS. Clinical practice. Preventing nephropathy induced by contrast medium. *N Engl J Med* 2006; **354**: 379-386

18 **Guevara M**, Fernández-Esparrach G, Alessandria C, Torre A, Terra C, Montañà X, Piera C, Alvarez ML, Jiménez W, Ginès P, Arroyo V. Effects of contrast media on renal function in patients with cirrhosis: a prospective study. *Hepatology* 2004; **40**: 646-651

19 **Najjar M**, Hamad A, Salameh M, Agarwal A, Feinfeld DA. The risk of radiocontrast nephropathy in patients with cirrhosis. *Ren Fail* 2002; **24**: 11-18

20 **Heyman SN**, Reichman J, Brezis M. Pathophysiology of radiocontrast nephropathy: a role for medullary hypoxia. *Invest Radiol* 1999; **34**: 685-691

21 **Brezis M**, Rosen S. Hypoxia of the renal medulla--its implications for disease. *N Engl J Med* 1995; **332**: 647-655

22 **Brezis M**, Agmon Y, Epstein FH. Determinants of intrarenal oxygenation. I. Effects of diuretics. *Am J Physiol* 1994; **267**: F1059-F1062

23 **Humes HD**, Hunt DA, White MD. Direct toxic effect of the radiocontrast agent diatrizoate on renal proximal tubule cells. *Am J Physiol* 1987; **252**: F246-F255

24 **Deray G**, Martinez F, Cacoub P, Baumelou B, Baumelou A, Jacobs C. A role for adenosine calcium and ischemia in radiocontrast-induced intrarenal vasoconstriction. *Am J Nephrol* 1990; **10**: 316-322

25 **Heyman SN**, Clark BA, Kaiser N, Spokes K, Rosen S, Brezis M, Epstein FH. Radiocontrast agents induce endothelin release in vivo and in vitro. *J Am Soc Nephrol* 1992; **3**: 58-65

26 **Baud L**, Ardaillou R. Reactive oxygen species: production and role in the kidney. *Am J Physiol* 1986; **251**: F765-F776

27 **Bakris GL**, Lass N, Gaber AO, Jones JD, Burnett JC Jr. Radiocontrast medium-induced declines in renal function: a role for oxygen free radicals. *Am J Physiol* 1990; **258**: F115-F120

28 **Baliga R**, Ueda N, Walker PD, Shah SV. Oxidant mechanisms in toxic acute renal failure. *Am J Kidney Dis* 1997; **29**: 465-477

29 **Mudge GH**. Uricosuric action of cholecystographic agents. A possible factor in nephrotoxicity. *N Engl J Med* 1971; **284**: 929-933

30 **Postlethwaite AE**, Kelley WN. Uricosuric effect of radiocontrast agents. A study in man of four commonly used preparations. *Ann Intern Med* 1971; **74**: 845-852

31 **Schwartz RH**, Berdon WE, Wagner J, Becker J, Baker DH. Tamm-Horsfall urinary mucoprotein precipitation by urographic contrast agents: in vitro studies. *Am J Roentgenol Radium Ther Nucl Med* 1970; **108**: 698-701

32 **Solomon R**, Werner C, Mann D, D'Elia J, Silva P. Effects of saline, mannitol, and furosemide to prevent acute decreases in renal function induced by radiocontrast agents. *N Engl J Med* 1994; **331**: 1416-1420

33 **Mueller C**, Buerkle G, Buettner HJ, Petersen J, Perruchoud AP, Eriksson U, Marsch S, Roskamm H. Prevention of contrast media-associated nephropathy: randomized comparison of 2 hydration regimens in 1620 patients undergoing coronary angioplasty. *Arch Intern Med* 2002; **162**: 329-336

34 **Merten GJ**, Burgess WP, Gray LV, Holleman JH, Roush TS, Kowalchuk GJ, Bersin RM, Van Moore A, Simonton CA 3rd, Rittase RA, Norton HJ, Kennedy TP. Prevention of contrast-induced nephropathy with sodium bicarbonate: a randomized controlled trial. *JAMA* 2004; **291**: 2328-2334

35 **Trivedi HS**, Moore H, Nasr S, Aggarwal K, Agrawal A, Goel P, Hewett J. A randomized prospective trial to assess the role of saline hydration on the development of contrast nephrotoxicity. *Nephron Clin Pract* 2003; **93**: C29-C34

36 **Mueller-Lenke N**, Buerkle G, Klima T, Breidthardt T, Buettner HJ, Mueller C. Incidence of contrast-induced nephropathy with volume supplementation--insights from a large cohort. *Med Princ Pract* 2008; **17**: 409-414

37 **Parfrey PS**, Griffiths SM, Barrett BJ, Paul MD, Genge M, Withers J, Farid N, McManamon PJ. Contrast material-induced renal failure in patients with diabetes mellitus, renal insufficiency, or both. A prospective controlled study. *N Engl J Med* 1989; **320**: 143-149

38 **Brar SS**, Shen AY, Jorgensen MB, Kotlewski A, Aharonian VJ, Desai N, Ree M, Shah AI, Burchette RJ. Sodium bicarbonate vs sodium chloride for the prevention of contrast medium-induced nephropathy in patients undergoing coronary angiography: a randomized trial. *JAMA* 2008; **300**: 1038-1046

39 **Ustundag Y**, Samsar U, Acikgoz S, Cabuk M, Kiran S, Kulah E, Aydemir S. Analysis of glomerular filtration rate, serum cystatin C levels, and renal resistive index values in cirrhosis patients. *Clin Chem Lab Med* 2007; **45**: 890-894

40 **Cholongitas E**, Shusang V, Marelli L, Nair D, Thomas M, Patch D, Burns A, Sweny P, Burroughs AK. Review article: renal function assessment in cirrhosis - difficulties and alternative measurements. *Aliment Pharmacol Ther* 2007; **26**: 969-978

41 **Pöge U**, Gerhardt T, Stoffel-Wagner B, Klehr HU, Sauerbruch T, Woitas RP. Calculation of glomerular filtration rate based on cystatin C in cirrhotic patients. *Nephrol Dial Transplant* 2006; **21**: 660-664

42 **Cárdenas A**, Ginès P, Uriz J, Bessa X, Salmerón JM, Mas A, Ortega R, Calahorra B, De Las Heras D, Bosch J, Arroyo V, Rodés J. Renal failure after upper gastrointestinal bleeding in cirrhosis: incidence, clinical course, predictive factors, and short-term prognosis. *Hepatology* 2001; **34**: 671-676

43 **Terra C**, Guevara M, Torre A, Gilabert R, Fernández J, Martín-Llahí M, Baccaro ME, Navasa M, Bru C, Arroyo V, Rodés J, Ginès P. Renal failure in patients with cirrhosis and sepsis unrelated to spontaneous bacterial peritonitis: value of MELD score. *Gastroenterology* 2005; **129**: 1944-1953

44 **Fraley DS**, Burr R, Bernardini J, Angus D, Kramer DJ, Johnson JP. Impact of acute renal failure on mortality in end-stage liver disease with or without transplantation. *Kidney Int* 1998; **54**: 518-524

45 **Chen YC**, Tsai MH, Hsu CW, Ho YP, Lien JM, Chang MY, Fang JT, Huang CC, Chen PC. Role of serum creatinine and prognostic scoring systems in assessing hospital mortality in critically ill cirrhotic patients with upper gastrointestinal bleeding. *J Nephrol* 2003; **16**: 558-565

46 **Bagshaw SM**, Gibney RT. Conventional markers of kidney function. *Crit Care Med* 2008; **36**: S152-S158

47 **Cramer BC**, Parfrey PS, Hutchinson TA, Baran D, Melanson DM, Ethier RE, Seely JF. Renal function following infusion of radiologic contrast material. A prospective controlled study. *Arch Intern Med* 1985; **145**: 87-89

48 **Heller CA**, Knapp J, Halliday J, O'Connell D, Heller RF. Failure to demonstrate contrast nephrotoxicity. *Med J Aust* 1991; **155**: 329-332

49 **Rao QA**, Newhouse JH. Risk of nephropathy after intravenous administration of contrast material: a critical literature analysis. *Radiology* 2006; **239**: 392-397

50 **Newhouse JH**, Kho D, Rao QA, Starren J. Frequency of serum creatinine changes in the absence of iodinated contrast material: implications for studies of contrast nephrotoxicity. *AJR Am J Roentgenol* 2008; **191**: 376-382

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Interleukin-1 and TNF-α polymorphisms and *Helicobacter pylori* in a Brazilian Amazon population

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Abstract

AIM: To study the association between Interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α polymorphisms, infection by *Helicobacter pylori* (*H pylori*) and the development of gastrointestinal diseases.

METHODS: Genomic DNA was extracted from the peripheral blood of 177 patients with various gastrointestinal diseases and from 100 healthy volunteers. The polymorphisms in IL-1β and TNF-α genes were analyzed using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) and those from IL-1RN with PCR. The presence of infection due to *H pylori* and the presence of the CagA toxin were detected by serology. The histopathological parameters in the gastric biopsies of the patients were according to the Sydney classification.

RESULTS: A comparison of the frequencies of the different polymorphisms studied among the patients and the control group demonstrated that the allele IL-1RN*2 was more frequent among patients with gastric ulcers and adenocarcinoma. Carriers of the allele IL-RN*2 and those with reactive serology for anti-CagA IgG had a greater risk of developing peptic ulcer and gastric adenocarcinoma, as well as a higher degree of inflammation and neutrophilic activity in the gastric mucosa.

CONCLUSION: Our results indicate a positive association between IL-1RN gene polymorphism and infection by positive *H pylori* CagA strains and the development of gastric ulcers and adenocarcinoma.

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Key words: *Helicobacter pylori*; Interleukin 1β gene; Interleukin-1 receptor antagonist gene; TNF-α gene; Cag pathogenicity island

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is the major cause of chronic superficial gastritis in humans, and is a hypochloridria etiological factor in the pathogenesis of peptic ulcer disease (PUD) and some forms of stomach

cancer[1]. However, most people harboring *H pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or stomach cancer[2]. The variability of clinical manifestations is associated with various factors, such as environmental, genetic susceptibility of the host and bacterial virulence[1,2].

One noteworthy factor for bacterial virulence is the CitoCagA toxin, codified by the *cagA* gene, a marker for the Cag-PAI pathogenicity island[3]. Infection by *H pylori* cagA-positive strains cause an intense inflammatory process, with dense neutrophilic infiltrate in the gastric mucosa[2,3]. However, bacterial virulence factors alone are not sufficient for determining clinical evolution of the infection. While virulent strains are frequent in both patients with peptic ulcers and those with gastric carcinoma[2,3], other factors in the host, mainly those that regulate immunological and inflammatory response may also contribute to a progression towards neoplasia[4].

Genetic polymorphisms, particularly those that occur in the region promoting genes that codify inflammatory cytokines, have been associated with an increase in synthesis of those interleukins and have emerged as a hypochloridria determining factor for cancer susceptibility[4,5].

Interleukin 1 beta (IL-1β) is a hypochloridria initiator and amplifier of the immune response, and is also a potent inhibitor of stomach acid secretion[5,6]. The antagonist of the IL-1 (IL-1RN) receptor is an anti-inflammatory cytokine that competes for the IL-1 receptors, modulating the effect of IL-1β[7]. Studies carried out in Caucasian and Asian populations have demonstrated that polymorphisms in genes IL-1β and IL-1RN are associated with an increased risk of hypochloridria and gastric carcinoma[7-9]. Another important cytokine is TNF-α, the biallelic polymorphism in position -308 of the region promoting the gene codifying that cytokine, which has been associated with the development of gastric carcinoma in studies carried out in Caucasians[10,11].

The objective of this study was to determine the frequency of polymorphisms in genes IL-1β, IL-1RN and TNF-α in patients from the state of Pará, in the northern region of Brazil, with various gastrointestinal diseases and in a control group, The relationship of these polymorphisms to infection by virulent strains of *H pylori* (CagA+) and to the histopathological characteristics of gastric tissue were also determined.

MATERIALS AND METHODS

Patient and control samples

Peripheral blood and gastric fragment samples were collected from 177 consecutive patients from the state of Pará-Brazil who had various gastrointestinal disturbances. Gastric fragment samples were obtained by the endoscopy service of the João de Barros Barreto Universitary Hospital. For the control samples, peripheral blood was collected from 100 patients who were without clinical or metabolic diseases and who were asymptomatic for gastrointestinal disturbances, and were thus not submitted to endoscopic examinations.

All patients and controls were enrolled in the study between September 2003 and September 2004 and were from the same socioeconomic level, and had similar cultural habits. All were natives of Pará state with the same ethnic background, approximately 50% Portuguese, 40% Amerindian and 10% African[12]. The study was approved by the Ethics Committee at João de Barros Barreto Universitary Hospital.

Detection of H pylori infection

In both patients and controls, the presence of the specific *H pylori* and CagA IgG antibodies in serum samples was determined. To detect *H pylori* antibodies the commercial HIK anti-*H pylori* EIA kit was used (Monobind, USA), and the Helicobacter P-120 EIA commercial kit (VIVA Diagnostica, Germany) was used to detect anti-CagA antibodies. Kits were used according to the manufacturers' technical descriptions.

IL-1β, IL-1RN and TNF-α genotyping

Genomic DNA was extracted from total blood using a leukocyte lysis solution (100 mmol/L Tris-HCl, 20 mmol/L EDTA, 200 mmol/L NaCl, 1% dodecyl-sodium sulfate, 0.2% β mercaptoethanol) and was purified using the phenol-chloroform method[13].

Polymorphisms of the IL-1β (-31, -511) and TNF-α (-308) genes were characterized using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). The volume for the PCR was 25 μL, containing 0.5 mmol/L of each primer, 1 X PCR buffer, 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of each nitrogenated base, 1.25 U of *Taq* DNA polymerase, 50 ng of DNA and sterile water.

To determine the polymorphism of the IL-1β gene in position -511, the PCR primers and conditions described by Wilkinson *et al*[14] were used. The PCR products were digested with *Ava*I overnight at 37℃ and separated by electrophoresis in 2% agarose gel stained with ethidium bromide. Those presenting two bands were called CC (114 and 190 bp), those with three bands, CT (114, 190 and 304 bp) and those with a single band, TT (304 bp).

Polymorphism of the IL-1β gene in the -31 position was investigated using the primers described by El-Omar *et al*[7]. The conditions of the PCR were as follows: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing and extension for 1 min, and final extension at 72℃ for 10 min. Annealing temperatures were set at 58℃ for primers. Negative and positive controls were used in all reactions. To discriminate alleles, the PCR products were digested with *Alu*I overnight at 37℃ and then separated by electrophoresis in 2% agarose gel stained with ethidium bromide. Those presenting a single band (235 bp) were called CC; those with three bands (98, 137 and 235 bp)

were called CT and TT was used for those with two bands (98 and 137 bp).

For genotyping polymorphism -308 in the TNF-α gene, the PCR primers and conditions as described by Wilson *et al*[15] were used. The PCR products were digested with *Nco*I overnight at 37℃ and separated by electrophoresis in 2% agarose gel stained with ethidium bromide.

The VNTR polymorphism at intron 2 of the IL-1RN gene was determined using the primers and conditions described by El-Omar *et al*[7]. The PCR products were separated by electrophoresis in 2% agarose gel stained with ethidium bromide. They were named allele 1 = 442 bp (4 repeats), allele 2 = 270 bp (2 repeats), allele 3 = 528 bp (5 repeats), allele 4 = 356 bp (3 repeats), allele 5 = 614 bp (6 repeats).

Histological evaluation

Biopsy specimens from the lesion and the adjacent area in each patient were obtained. The specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4-μm sections, and stained with hematoxylin and eosin (HE). The histopathological parameters were graded (0-3) using the criteria described in the updated Sydney classification system[16] for analysis of chronic inflammation, polymorphonuclear activity, and intestinal metaplasia.

Statistical analysis

Hardy-Weinberg equilibrium and heterogeneity among groups were tested using the Guo and Thompson[17] exact test. Maximum-likelihood haplotype frequencies were computed using an Expectation-Maximization (IN) algorithm[18,19]. Linkage disequilibrium was tested using a likelihood-ratio test[20]. All the aforementioned statistical procedures were carried out using Arlequin software[21].

To compare the variables of sex, age, CagA status, *H pylori* and genotype frequencies between patients and controls, the G test was utilized. The risks of carriers with different alleles developing gastric ulcers and adenogastric carcinoma, as well as having alterations in the gastric mucosa were calculated using the Odds ratio. The data were analyzed with BioEstat version 4.0 software[22]. Differences were considered statistically significant if *P* values were less than 0.05.

RESULTS

Clinical and demographic characteristics of patients and controls

One hundred and seventy-seven patients with various gastrointestinal pathologies were investigated; of these 80 (45%) had gastritis, 33 (19%) duodenal ulcer, 34 (19%) gastric ulcer and 30 (17%) had intestinal-type adenogastric carcinoma.

The epidemiological data of the two groups studied are described in Table 1. The patients had an average age of 45 years with ages ranging from 18 to 90 years. Subjects in the control group had an average age of 36 years, with ages ranging from 18 to 81 years. The patients with adenogastric carcinoma were older than those in the control group (Table 1).

Table 1 Epidemiological characteristics of the control group and patients *n* (%)

Demographic data	Control *n* = 100	Gastritis *n* = 80	DU *n* = 33	GU *n* = 34	AC *n* = 30
Age (yr)					
> 50	31	26 (32)	11 (33)	12 (35)	21 (70)
< 50	69	54 (68)	22 (67)	22 (65)	9 (30)
P value		NS	NS	NS	0.00
Sex					
Male	57	39 (49)	19 (58)	21 (62)	19 (63)
Female	43	41 (51)	14 (42)	13 (38)	11 (37)
P value		NS	NS	NS	NS
IgG-anti *H pylori*					
Positive	61	74 (92)	30 (91)	33 (97)	29 (97)
Negative	39	6 (8)	3 (9)	1 (3)	1 (3)
P value		0.00	0.00	0.00	0.00
anti-CagA IgG (HP+)					
Positive	38	53 (66)	29 (97)	31 (94)	28 (97)
Negative	23	21 (34)	1 (3)	2 (6)	1 (3)
P value		NS	0.00	0.00	0.00

DU: Duodenal ulcer; GU: Gastric ulcer; AC: Adenogastric carcinoma; G Test: Control *versus* disease; NS: Not significant.

The presence of IgG antibodies-*H pylori* and CagA specific was greater in patients with gastrointestinal diseases than in the control group (Table 1). A comparison of the presence of anti-CagA antibodies IgG in patients with various gastrointestinal diseases demonstrated that patients with gastric ulcer (G = 6.330, *P* = 0.011), duodenal ulcer (G = 8.076, *P* = 0.004) and adenogastric carcinoma (G = 7.702, *P* = 0.005) had greater seroprevalence of this antibody that patients with gastritis. However, when we compared the frequency observed in patients with gastric ulcer with that in patients with duodenal ulcer (G = 0.007, *P* = 0.932) and with stomach cancer (G = 0.013, *P* = 0.908) no differences were observed. A similar observation was made when we compared patients with duodenal ulcer and those with cancer (G = 0.506, *P* = 0.476).

IL-1β, IL-1RN and TNF-α polymorphisms

The polymorphisms studied were in the Hardy-Weinberg equilibrium for both the control group (IL-1β-31 *P* = 0.811; IL-1β-511 *P* = 0.902; IL-1RN *P* = 0.361; TNF-α-308 *P* = 0.363) and for patients with gastrointestinal diseases (IL-1β-31 *P* = 0.321; IL-1β-511 *P* = 0.791; IL-1RN *P* = 0.691; TNF-α-308 *P* = 0.552). An imbalance in linkage among alleles IL-1β-511T and IL-1β-31C ($P > 10^{-5}$) was observed in both groups studied.

A comparison of the genotype frequencies of polymorphisms in genes IL-1β analyzed in this study with those from other studies carried out in different countries, demonstrated that the frequency of the alleles for polymorphisms IL-1β-31, IL-1β-511 in our population did not differ statistically from that described for Caucasians[7] and Asiatic populations[23], and was

Table 2 Comparison of genotype frequencies for the polymorphisms of IL-1β genes studied with results of other studies in different ethnic and population groups *n* (%)

Genotypes	Pará (Brazil)	Minas Gerais (Brazil)	Caucasians	Asians
IL-1β-31				
TT	9	102 (35.8)[1]	7 (12)[2]	7 (4)[4]
TC	23	138 (48.4)	21 (36)	34 (20)
CC	68	45 (15.8)	30 (52)	128 (76)
P value	Reference	0.001	NS	NS
IL-1β-511				
CC	31	108 (37.9)[1]	29 (50)[2]	34 (20)[4]
CT	46	137 (48.1)	21 (36)	97 (57)
TT	23	40 (14)	8 (14)	38 (23)
P value	Reference	NS	NS	NS
IL-1RN				
11	58	175 (61.4)[1]	37 (64)[2]	163 (96)[4]
12	36	92 (32.3)	14 (24)	4 (3)
22	6	18 (6.3)	17 (10)	2 (1)
P value	Reference	NS	NS	0.001
TNF-α-308				
GG	86	223 (78.3)[1]	152 (72)[3]	274 (91.3)[5]
GA	13	54 (18.9)	52 (25)	24 (8)
AA	1	8 (2.8)	6 (3)	2 (0.7)
P value	Reference	NS	0.025	NS

G Test: Our study *versus* other papers. [1]Queiroz *et al*[25] 2004; [2]El-Omar *et al*[7] 2000; [3]El-Omar *et al*[24] 2003; [4]Zeng *et al*[23] 2003; [5]Yea *et al*[11] 2001. NS: Not significant.

Table 3 Distribution of genotypes for *IL-1β* (-511 and -31), *IL-1RN* and TNF-α in the control group and in patients with gastrointestinal pathologies *n* (%)

Genotypes	Control *n* = 100	Gastritis *n* = 80	DU *n* = 33	GU *n* = 34	AC *n* = 30
IL-1β-31					
T/T	9	3 (4)	1 (3)	2 (6)	1 (4)
C/T	23	21 (26)	9 (27)	7 (20)	4 (13)
C/C	68	56 (70)	23 (70)	25 (74)	25 (83)
P value		NS	NS	NS	NS
IL-1β-511					
C/C	31	26 (33)	9 (27)	10 (29)	6 (20)
C/T	46	34 (42)	13 (39)	14 (41)	11 (37)
T/T	23	20 (25)	11 (34)	10 (30)	13 (43)
P value		NS	NS	NS	NS
IL-1RN					
1/1	58	41 (51)	15 (46)	11 (32)	10 (33)
1/2	36	34 (43)	16 (49)	21 (62)	19 (63)
2/2	6	5 (6)	2 (6)	2 (6)	1 (4)
P value		NS	NS	0.00	0.00
TNF-α					
G/G	86	62 (77)	23 (70)	29 (85)	24 (80)
G/A	13	16 (20)	8 (24)	4 (12)	5 (16)
A/A	1	2 (3)	2 (6)	1 (3)	1 (4)
P value		NS	NS	NS	NS

DU: Duodenal ulcer; GU: Gastric ulcer; AC: Adenogastric carcinoma; G Test: Control *versus* disease; NS: Not significant.

in an intermediate position compared to that found in those populations. In contrast, the polymorphisms of the IL-1RN genes differed from those described in Asiatic populations[11] and gene TNF-α-308 differed from descriptions in Caucasian populations[24] (Table 2). When we compared our data with those obtained in Minas Gerais[25], we observed a difference in the frequency of the IL-1β-31 polymorphism, with the C allele being more frequent in our population than in the Minas Gerais population (Table 2).

In analyzing the distribution of the different genotypes among the patients and the control group we observed that the polymorphisms in genes IL-1β-31, IL-1β-511, TNF-α-308 did not differ. However, in relation to gene IL-1RN, we obtained a greater frequency of allele 2 carriers (IL-1RN*2) among patients with adenogastric carcinoma and gastric ulcer than in the control group (Table 3).

A combined risk analysis of the different polymorphisms studied demonstrated that there was no synergism between those polymorphisms and the development of gastric ulcers and adenocarcinoma. Individuals carrying only a polymorphism in gene IL-1RN*2 (OR = 3, $P = 0.86$) had a greater risk than individuals carrying polymorphisms in genes IL-31*C and IL-1RN*2 (OR = 1.2, $P = 0.51$) and the risk was similar to that for carriers of all the polymorphisms studied (OR = 3, $P = 0.71$).

In the association between the IL1-RN polymorphism and the presence of specific CagA antibodies, we found that carriers of allele IL-1RN*2 who were reactive for anti-CagA IgG had a greater risk of developing gastric ulcers and adenocarcinoma. This demonstrated an interaction between the presence of a virulent strain and allele IL-RN*2 in the development of these diseases (Table 4).

The relationship between IL-1RN genotypes and anti-CagA antibodies with histopathological data demonstrated that carriers of allele IL-1RN*2, who were seroreactive for CagA had high levels of inflammation and neutrophilic activity, with a heightened risk of developing intestinal metaplasia in the gastric mucosa (Table 5).

DISCUSSION

The state of Pará has a high prevalence and incidence of gastrointestinal diseases, principally adenogastric carcinoma. In addition, an increased prevalence of *H pylori* infection has been observed among patients with gastrointestinal diseases[26,27], with a predominance of virulent strains (*vacA*-s1b/m1/*cagA*-positive) that are associated with the development of both peptic ulcers and adenocarcinoma[28].

The patients with adenogastric carcinoma were older than those in the control group. These patients had been developing progressive gastric cancer lesions for a long time[29].

Some studies have demonstrated that polymorphisms in genes IL-1β, IL-1RN and TNF-α, together with *H pylori* infection are associated with an increased risk of developing stomach cancer[7,10,11]; therefore, to better understand the factors related to the high prevalence of stomach cancer in our region we analyzed the frequency of the genotypes of polymorphisms in genes IL-1β,

Table 4 Combined risk of polymorphism in the IL1-RN gene and IgG CagA antibody for development of gastric ulcers and adenogastric carcinoma

IL1-RN	CagA	Control	GU	OR[a] (95% IC)	*P*	AC	OR[b] (95% IC)	*P*
1/1	(-)	15 (24%)	2 (6%)	Ref.	-	1 (4%)	Ref.	-
2* carrier	(-)	8 (13%)	2 (6%)	1.875[c]	0.983	1 (4%)	1.875[c]	0.735
1/1	(+)	21 (35%)	9 (27%)	3.214 (0.605-17.063)	0.289	8 (30%)	5.714 (0.644-50.648)	0.185
2* carrier	(+)	17 (28%)	20 (61%)	8.823 (1.762-44.181)	0.008	19 (62%)	16.764 (1.997-140.707)	0.004
Total		61	33			29		

GU: Gastric ulcer; AC: Adenogastric carcinoma. [a]Control x GU; [b]Control x AC; [c]The confidence interval was not calculated, since, $n_1p_1q_1 < 5$ or $n_2p_2q_2 < 5$.

Table 5 Association of polymorphism for IL-RN gene and anti-CagA antibodies with histopathological findings from patients

IL1-RN	CagA	DI		OR (95% CI)	*P*	NA		OR (95% CI)	*P*	Metaplasia		OR (95% CI)	*P*
		1	2 and 3			1	2 and 3			+	-		
1/1	(-)	13	2	-	-	12	3	-	-	1	14	-	-
2* carrier	(-)	9	1	0.722[a]	0.706	8	2	1[a]	0.609	2	8	3.5[a]	0.706
1/1	(+)	24	34	9.2 00 (1.90-44.606)	0.004	26	32	4.923 (1.254-19.314)	0.032	17	41	5.804 (0.706-47.693)	0.139
2* carrier	(+)	20	63	20.475 (4.253-98.556)	0.001	23	60	10.434 (2.695-40.388)	0.003	35	58	8.448 (1.064-67.065)	0.038

DI: Degree of inflammation; NA: Neutrophilic activity. Histopathological parameters: 1: Light; 2: Moderate; 3: Intense. [a]The confidence interval was not calculated, since, $n_1p_1q_1 < 5$ or $n_2p_2q_2 < 5$.

IL-1RN and TNF-α and the presence of infection by CagA+ strains in patients with various gastrointestinal diseases and in a control group.

In this study, infection by virulent strains (CagA+) was greater in patients with peptic ulcers and adenocarcinoma than in patients with gastritis or in subjects in the control group. Similar results were found in a previous study carried out in Belém and other Brazilian states, where the presence of *H pylori* CagA+ strains was associated with the development of peptic ulcers and adenocarcinoma[28,29].

The frequency of polymorphisms in genes IL-1β-31 and IL-1β-511 in our study was similar to that described in Caucasian[7,24] and Asiatic populations[23,30], whereas the frequency of polymorphisms in genes IL-1RN and TNF-α-308 was significantly different from that reported in Asiatic[23] and Caucasian[7,24] populations, respectively. The genetic composition of the Brazilian population is made up of a genetic mix of various ethnic groups, including Portuguese, Africans and Amerindians[12]. The differences and similarities between the allelic frequencies of the polymorphisms studied in our population with those of other ethnic groups are products of the genetic mix that has occurred in Brazil.

In comparing the frequencies of the polymorphisms studied in our population with those from another Brazilian study carried out in Belo Horizonte, Minas Gerais, located in the central-western region of the country, we observed differences in relation to IL-1β-31 polymorphism. In Brazil, several states show differences in ethnic background, and the populations in the Amazon region are the ones which have an important indigenous genetic component[12], greater than that described for the Minas Gerais population[31], which may be reflected in the gene frequency of those polymorphisms.

An analysis of the genotypes of the polymorphisms in the patients and the control group demonstrated that IL-1RN*2 carriers were more frequent among patients with gastric ulcers and adenocarcinoma. The IL-1Ra protein (codified by the IL-1RN gene) acts competitively to inhibit action by IL-1β[7]. Carriers of IL-1RN*2 have higher levels of IL-1β in the gastric mucosa than those with IL-1RN1/1[32], and thus have a more severe and prolonged immune response, which may lead to hypochloridria due to destruction of the gastric glands and to action by IL-1β that inhibits synthesis of chloridic acid by the parietal cells[6,7]. El-Omar *et al*[7] have described an association between IL-1RN*2 and hypochloridria, and both gastric ulcer and stomach cancer reduce the synthesis of chloridic acid. Other studies have also described the association between the IL-1RN*2 polymorphisms and stomach cancer[23,24,33]. In Brazil, Rocha *et al*[34] obtained similar results in relation to the association of allele IL-RN*2 and an increased risk of developing stomach cancer, as well as the absence of an association between the IL-1β-31, TNF-α-308 polymorphisms and the risk of developing stomach cancer.

A combined analysis of the different polymorphisms demonstrated that there was no association between these polymorphisms and an increased risk of developing gastric ulcer or adenocarcinoma. In addition, we found an interaction between the presence of allele IL-RN*2 and infection by CagA+ strains. This finding is important for our region, which has a high incidence of stomach cancer, high prevalence of infection by CagA+

strains and a high frequency of the IL-1RN*2 allele. Reinforcing these data, our results have demonstrated that carriers of allele IL-RN*2 infected by CagA+ strains had a greater risk of developing an intense inflammatory process in the gastric mucosa, which confirms a synergistic action between the polymorphism of gene IL-1RN and another type of infecting strain. Other studies have also observed that both infection by virulent strains and gene IL-1RN polymorphism are important risk factors for gastric carcinogenesis[23,29,33]. In conclusion, our results suggest that bacterial virulence and genetic factors in the host act synergistically in the development of gastric ulcers and adenocarcinoma.

COMMENTS

Background

Helicobacter pylori (*H pylori*) infection is associated with a broad spectrum of gastrointestinal disorders. However, most people harboring *H pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or stomach cancer. The variability of clinical manifestations is associated with various factors, such as environmental, genetic susceptibility of the host and bacterial virulence.

Research frontiers

This study indicated a possible association between IL-1RN gene polymorphism and infection by positive *H pylori* CagA strains and the development of gastric ulcers and adenocarcinoma.

Innovations and breakthroughs

This study determined the frequency of polymorphisms in genes IL-1β, IL-1RN and TNF-α in patients with various gastrointestinal diseases from the state of Pará, in the Brazilian Amazon, and in a control group. The relationship of these polymorphisms to infection by virulent strains of *H pylori* and to the histopathological characteristics of the gastric tissue were determined.

Applications

This study may represent a future strategy for distinguishing patients with a risk of developing gastrointestinal diseases, such as gastric cancer.

Terminology

The CitoCagA toxin is a factor for bacterial virulence. The antagonist of the IL-1 (IL-1RN) receptor is an anti-inflammatory cytokine.

Peer review

In this study, confirmatory new data on gene polymorphism in a Brazilian population and message is clearly shown.

REFERENCES

1 **Shiotani A**, Nurgalieva ZZ, Yamaoka Y, Graham DY. Helicobacter pylori. *Med Clin North Am* 2000; **84**: 1125-1136, viii

2 **Atherton JC**, Peek RM Jr, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* 1997; **112**: 92-99

3 **Segal ED**, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc Natl Acad Sci USA* 1999; **96**: 14559-14564

4 **Perez-Perez GI**, Garza-Gonzalez E, Portal C, Olivares AZ. Role of cytokine polymorphisms in the risk of distal gastric cancer development. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 1869-1873

5 **El-Omar EM**. The importance of interleukin 1beta in Helicobacter pylori associated disease. *Gut* 2001; **48**: 743-747

6 **Wolfe MM**, Nompleggi DJ. Cytokine inhibition of gastric acid secretion--a little goes a long way. *Gastroenterology* 1992; **102**: 2177-2178

7 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398-402

8 **Hwang IR**, Kodama T, Kikuchi S, Sakai K, Peterson LE, Graham DY, Yamaoka Y. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1beta production in Helicobacter pylori infection. *Gastroenterology* 2002; **123**: 1793-1803

9 **Machado JC**, Pharoah P, Sousa S, Carvalho R, Oliveira C, Figueiredo C, Amorim A, Seruca R, Caldas C, Carneiro F, Sobrinho-Simões M. Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. *Gastroenterology* 2001; **121**: 823-829

10 **Shibata J**, Goto H, Arisawa T, Niwa Y, Hayakawa T, Nakayama A, Mori N. Regulation of tumour necrosis factor (TNF) induced apoptosis by soluble TNF receptors in Helicobacter pylori infection. *Gut* 1999; **45**: 24-31

11 **Yea SS**, Yang YI, Jang WH, Lee YJ, Bae HS, Paik KH. Association between TNF-alpha promoter polymorphism and Helicobacter pylori cagA subtype infection. *J Clin Pathol* 2001; **54**: 703-706

12 **Batista dos Santos SE**, Rodrigues JD, Ribeiro-dos-Santos AK, Zago MA. Differential contribution of indigenous men and women to the formation of an urban population in the Amazon region as revealed by mtDNA and Y-DNA. *Am J Phys Anthropol* 1999; **109**: 175-180

13 **Blin N**, Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res* 1976; **3**: 2303-2308

14 **Wilkinson RJ**, Patel P, Llewelyn M, Hirsch CS, Pasvol G, Snounou G, Davidson RN, Toossi Z. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1beta on tuberculosis. *J Exp Med* 1999; **189**: 1863-1874

15 **Wilson AG**, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci USA* 1997; **94**: 3195-3199

16 **Dixon MF**, Genta RM, Yardley JH, Correa P. Histological classification of gastritis and Helicobacter pylori infection: an agreement at last? The International Workshop on the Histopathology of Gastritis. *Helicobacter* 1997; **2** Suppl 1: S17-S24

17 **Guo SW**, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 1992; **48**: 361-372

18 **Excoffier L**, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 1995; **12**: 921-927

19 **Lange K**. Mathematical and statistical methods for genetic analysis. 1st ed. New York: Springer, 1997: 39-52

20 **Slatkin M**, Excoffier L. Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. *Heredity* 1996; **76** (Pt 4): 377-383

21 **Schneider S**, Roessli D, Excoffier L. Arlequin Ver 2.0: A Software for population genetics data analysis. Genetics and Biometry Lab, Department of Anthropology, University of Geneva, Switzerland. 2000

22 **Ayres M**, Ayres Jr M, Ayres DL, dos Santos AS. Bioestat 4.0: Aplicações estatísticas nas áreas das ciências biológicas e médicas. 3rd ed. Belém: MCT-CNPq, 2005: 125-139

23 **Zeng ZR**, Hu PJ, Hu S, Pang RP, Chen MH, Ng M, Sung JJ. Association of interleukin 1B gene polymorphism and gastric cancers in high and low prevalence regions in China. *Gut* 2003; **52**: 1684-1689

24 **El-Omar EM**, Rabkin CS, Gammon MD, Vaughan TL, Risch HA, Schoenberg JB, Stanford JL, Mayne ST, Goedert J, Blot WJ, Fraumeni JF Jr, Chow WH. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* 2003; **124**: 1193-1201

25 **Queiroz DM**, Guerra JB, Rocha GA, Rocha AM, Santos A,

De Oliveira AG, Cabral MM, Nogueira AM, De Oliveira CA. IL1B and IL1RN polymorphic genes and Helicobacter pylori cagA strains decrease the risk of reflux esophagitis. *Gastroenterology* 2004; **127**: 73-79

26 **Aguiar DC**, Corvelo TC, Ara jo M, Cruz EM, Daibes S, Assumpção MB. [Expression of ABH and Lewis antigens in chronic gastritis and pre-neoplasic alterations in gastric mucosa] *Arq Gastroenterol* 2002; **39**: 222-232

27 **Martins LC**, Corvelo TC, Oti HT, Barile KA. [Seroprevalence of antibodies against Helicobacter pylori CagA antigen in patients with gastric ulcer in the North region of Brazil] *Rev Soc Bras Med Trop* 2002; **35**: 307-310

28 **Martins LC**, Corvelo TC, Demachki S, Araujo MT, Assumpção MB, Vilar SC, Freitas FB, Barbosa HP, Fecury AA, do Amaral RK, Dos Santos SE. Clinical and pathological importance of vacA allele heterogeneity and cagA status in peptic ulcer disease in patients from North Brazil. *Mem Inst Oswaldo Cruz* 2005; **100**: 875-881

29 **Brito CA**, Silva LM, Jucá N, Leal NC, de Souza W, Queiroz D, Cordeiro F, Silva NL. Prevalence of cagA and vacA genes in isolates from patients with Helicobacter pylori-associated gastroduodenal diseases in Recife, Pernambuco, Brazil. *Mem Inst Oswaldo Cruz* 2003; **98**: 817-821

30 **Guo W**, Wang N, Li Y, Zhang JH. Polymorphisms in tumor necrosis factor genes and susceptibility to esophageal squamous cell carcinoma and gastric cardiac adenocarcinoma in a population of high incidence region of North China. *Chin Med J* (Engl) 2005; **118**: 1870-1878

31 **Parra FC**, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. *Proc Natl Acad Sci USA* 2003; **100**: 177-182

32 **Vilaichone RK**, Mahachai V, Tumwasorn S, Wu JY, Graham DY, Yamaoka Y. Gastric mucosal cytokine levels in relation to host interleukin-1 polymorphisms and Helicobacter pyloricagA genotype. *Scand J Gastroenterol* 2005; **40**: 530-539

33 **Chen A**, Li CN, Hsu PI, Lai KH, Tseng HH, Hsu PN, Lo GH, Lo CC, Lin CK, Hwang IR, Yamaoka Y, Chen HC. Risks of interleukin-1 genetic polymorphisms and Helicobacter pylori infection in the development of gastric cancer. *Aliment Pharmacol Ther* 2004; **20**: 203-211

34 **Rocha GA**, Guerra JB, Rocha AM, Saraiva IE, da Silva DA, de Oliveira CA, Queiroz DM. IL1RN polymorphic gene and cagA-positive status independently increase the risk of noncardia gastric carcinoma. *Int J Cancer* 2005; **115**: 678-683

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BRIEF ARTICLES

Primary epithelial tumours of the appendix in a black population: A review of cases

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Abstract

AIM: To determine the prevalence, histologic types and clinical features of primary epithelial tumours of the vermiform appendix in a predominantly black population.

METHODS: All cases of primary tumours of the appendix identified by review of the histopathology records at the University of the West Indies between January 1987 and June 2007 were selected. Relevant pathologic and clinical data were extracted with supplementation from patient charts where available. Non-epithelial tumours were excluded. The total number of appendectomy specimens over the period was also ascertained.

RESULTS: Forty-two primary epithelial tumours were identified out of 6 824 appendectomies yielding a prevalence rate of approximately 0.62%. Well-differentiated neuroendocrine cell tumours (carcinoids, 47.6%) and benign non-endocrine cell tumours (adenomas, 45.2%) were most common with nearly equal frequency. The median age was 43 years, with no sex predilection. Carcinoid tumours occurred in younger patients (mean age 32 years), with a male-to-female ratio of 1.2:1. A clinical diagnosis of acute appendicitis was the most common reason for appendectomy (57.1%) and was histologically confirmed in 75% (18 of 24) of cases. In total, 16.7% of cases were diagnosed after incidental appendectomy.

CONCLUSION: Appendiceal epithelial tumours are rare in our experience, and are represented principally by carcinoid tumours and adenomas. Carcinoid tumours occurred in younger patients but were slightly more common in men than women. Tumours were not suspected clinically and were diagnosed incidentally in specimens submitted for acute appendicitis supporting the need for histological evaluation in all resection specimens.

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Key words: Epithelial tumours; Appendiceal tumours; Carcinoid; Adenoma; Appendicitis

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INTRODUCTION

Primary appendiceal tumours are uncommon. They are often diagnosed incidentally after histopathological examination of the vermiform appendix submitted in the course of the management of another clinical diagnosis. This paper reviews the primary appendiceal tumours diagnosed at the University Hospital of the West Indies during the period from January 1987 to June 2007, and to our knowledge represents the first such analysis in a predominantly black population.

MATERIALS AND METHODS

The surgical pathology records of the Department of Pathology at the University of the West Indies were retrospectively reviewed over the time period January 1987-June 2007 and all cases of primary neoplasms of the appendix were selected. Non-epithelial tumours were excluded from the study population because of diagnostic controversy in the absence of immunohistochemical

Table 1 Demographic data and histologic types

Tumour type	Age range (yr)	Mean age (yr)	Median age (yr)
Carcinoid	18-70	32 ± 20.3	35.8
Adenoma	29-87	59 ± 15.2	58.6
Adenocarcinoma	36-74	55 ± 19.0	55

evaluation. The parameters examined included patient age, sex, the clinical history, the surgical procedure, the gross description of the specimen submitted and the histopathological diagnosis. Patient charts were retrieved where possible, for additional clinical information. The total number of appendectomies over the time period was also ascertained.

RESULTS

In the period under study, 6824 appendices were submitted for pathologic evaluation. Forty-two primary epithelial tumours of the appendix were identified, yielding a prevalence rate of approximately 0.62%.

The overall age range of patients was 18-87 years with a mean age of 45.9 ± 19.3 years and a median age of 43 years. The male-to-female ratio for all tumours was 1:1. Table 1 illustrates the demographic data with respect to each tumour type.

Of the 42 epithelial tumours identified, 20 (47.6%) were carcinoids, including 1 insular and 3 goblet cell carcinoids. Of the 19 (45.2%) adenomas, we identified 15 villous adenomas, the majority of which (11, 26.2%) were morphologically mucinous cystadenomas, as well as 1 tubular and 3 tubulovillous adenomas. All 3 cases of adenocarcinoma were of the mucinous type. Interestingly no cases of serrated adenomas were found in the records.

Only in 3 cases of carcinoid and 1 case of mucinous cystadenocarcinoma was the lesion identified grossly. Measurements were recorded for 2 out of 3 cases of carcinoid measuring 1.3 and 1 cm in maximum dimension.

In 26 of the cases the clinical diagnosis was acute appendicitis, appendiceal abscess in 3, appendix mass, caecal carcinoma, primary peritonitis and an appendiceal tumour in 1 case each. In a further 7 cases, the vermiform appendix was removed incidentally. No clinical diagnosis was submitted in 2 cases.

Of the 42 cases, only 7 patient charts were retrievable; review of the clinical notes revealed aggressive behaviour in one case of carcinoid tumour (goblet cell variant) with hepatic metastases, in a 50-year-old female, who presented with perforated appendicitis. The tumour was not identified grossly in this case.

In 4 of 42 cases there were synchronous colonic neoplasms giving a prevalence rate of 9.5%. Two of these 4, were cases of solitary synchronous caecal carcinoma accompanying a mucinous cystadenoma of the appendix in 1 case and an adenocarcinoma of the appendix in the other. In the other 2 cases, there was a single case of a caecal carcinoma and 2 tubulovillous and 1 villous colonic adenomas coexistent with an appendiceal tubulovillous adenoma in a right hemicolectomy specimen. The other case was that of a caecal carcinoma and 2 tubulovillous colonic adenomas coexistent with a mucinous cystadenoma of the appendix in a right hemicolectomy specimen. The carcinoma was diagnosed pre-operatively in 3 of the cases with no pre-operative diagnosis proffered in the other case. In none of these 4 cases was an appendiceal tumour suspected, nor was there evidence of any inherited syndromes in any of the cases.

No metachronous colorectal lesions were diagnosed up to June 2007. This search was limited by the retrieval of only 7 patient charts.

Pseudomyxoma peritonei was rare, with 1 such case diagnosed in a patient with a mucinous cystadenoma. Acute appendicitis was found to complicate a neoplasm in 54.8% of cases. Incidental appendectomy contributed to 16.7% of the neoplasms diagnosed.

DISCUSSION

Tumours of the appendix are uncommon. Our prevalence of 0.62% is comparable to the prevalence rate of 0.5%-0.9% found in other studies in New Zealand and the United Kingdom[1-3]. No published reports are available for other predominantly black populations. The clinical picture was most frequently that of acute appendicitis (24 of 42) with histologic confirmation in 75% of these cases. Of note, incidental appendectomy provided 7 cases (16.7%). This highlights the utility of routine histopathologic examination of appendiceal specimens because the diagnosis is often made without antecedent clinical suspicion and these diagnoses can potentially alter patient management[4].

Importantly, the index of suspicion of appendiceal tumours should be raised in cases clinically suggestive of acute appendicitis in the middle aged and elderly, given the median age of 43 at diagnosis of these tumours in our experience.

Carcinoid tumours show epithelial and neuroendocrine differentiation, and may arise in many sites, but most commonly in the gastrointestinal tract[5]. These tumours frequently arise at the tip of the appendix[6] and are reportedly the most common tumours of the appendix[2,7]. Gaskin *et al*[8] in a previous study of carcinoids of the gastrointestinal tract from our institution reported that the appendix was the most common location with a similar mean age to that documented in this study. In our study, there were almost equal numbers of carcinoids and adenomas, but patients with carcinoids were younger than those with adenomas and adenocarcinomas. There is a paucity of literature with regards to neuroendocrine tumours in black populations, however, in one study the incidence of carcinoid tumours in all sites was found to be highest in African-American males[9]. Other studies, including those from the SEER data (1973-2001) report that appendiceal neuroendocrine tumours are more common in females[8,10,11], in contrast to our data, which revealed a slight male predilection. This may be due in part to the small size of the study population.

While the overall behaviour of carcinoids is unpredictable, appendiceal carcinoids have an excellent prognosis[5,11]. Importantly, where lesions are identified in the gross assessment of the specimen, they should be measured. Appendectomy is appropriate for lesions

< 1 cm but for lesions over 2 cm in diameter there is a significant increase in metastatic spread and thus right hemicolectomy is required in such cases[3,6]. There remains controversy around what is the appropriate treatment for lesions between 1-2 cm. Authors have suggested that additional criteria be examined in such cases. These criteria include proliferation markers, mitotic activity, vascular and mesoappendiceal invasion[6,12]. Unfortunately, there was one case of an unmeasured grossly visible lesion in our review. This potentially exposed the patient to not receiving further surgery which may have been necessary.

After diagnosis of an epithelial non-endocrine neoplasm of the appendix, the entire colorectum should be examined for synchronous lesions[13,14]. In this series, appendiceal adenomas were associated with synchronous colonic tumours in 9.5% of cases, further underscoring the need for colorectal examination and surveillance post diagnosis of appendiceal adenoma. The relative unavailability of colonoscopy in our population over the period and the small sample size may be responsible for such a high rate when compared to Khan *et al*[14]. The absence of cases of serrated adenomas may reflect past alternative classification of these lesions, or a lack of reporter awareness or it may be highlighting a lesser contribution by the serrated pathway to colorectal carcinoma in our population. This area requires further study. Based on the available records, there were no cases of colorectal carcinoma syndromes with appendiceal involvement diagnosed during the period. Pseudomyxoma peritonei, a term best avoided for diagnostic purposes, was extremely rare.

COMMENTS

Background

Primary appendiceal tumours are uncommon. They are often diagnosed incidentally after histopathological examination of the vermiform appendix submitted in the course of the management of another clinical diagnosis. No previous research has been done in a predominantly black population to determine the prevalence or histologic types of primary epithelial tumours of the appendix.

Research frontiers

Carcinoids (neuroendocrine tumours) are considered the most common primary appendiceal neoplasm and there is some evidence that African-American males have the highest incidence of carcinoids in all sites. However, several articles including the SEER database identified carcinoids more frequently in females. The roles of gender and ethnicity, if any, are unclear and require further investigation. The serrated pathway to colorectal carcinoma has been well described but the absence of such lesions in this study may reflect inappropriate classification or lack of reporter awareness. Further specific research into the serrated pathway to colorectal carcinoma in predominantly black populations is required.

Innovations and breakthroughs

This and other articles have validated the use of routine histologic examination of appendicectomy specimens given the frequency of diagnosis of incidental tumours which potentially require specific management. Generally carcinoid tumours portend a favourable prognosis but in some cases these tumours behave aggressively. Several criteria including proliferative markers, mitotic activity, vascular and mesoappendiceal invasion are being examined to determine their utility in predicting the behaviour of carcinoid tumours. The gross documentation of tumour size is important in determining if further management is required. While carcinoids are generally thought to be the most common tumour of the appendix, in this study an almost equal number of adenomas were diagnosed. The published description of the sessile serrated adenoma and its recognition as a precursor to adenocarcinoma of the colon and rectum opened a new paradigm of research into colorectal carcinoma. It also highlighted that these lesions may have been previously misdiagnosed as hyperplastic polyps.

Applications

Routine histopathological examination of appendicectomy specimens is justified given the not infrequent incidental finding of appendiceal tumours. In cases of appendicitis in the elderly, the index of suspicion for epithelial tumours of the appendix should be raised. Moreover, once the diagnosis of an adenomatous lesion is made, colonoscopic examination of the entire large bowel is mandatory given the frequency of synchronous colorectal neoplasia in our population.

Terminology

The term carcinoid refers to tumours which show epithelial, endocrine and neural characteristics which have been identified by light microscopic, ultrastructural and immunohistochemical means. The term serrated pathway of colorectal carcinoma refers to those lesions which arise from precursors with a peculiar hyperplastic morphology associated with architectural dysplasia but no cytologic abnormalities. These tumours have specific mutations which further distinguish them from the adenomatous and the hereditary nonpolyposis colorectal cancer (HNPCC) pathway.

Peer review

This retrospective brief report is the first publication describing the prevalence and types of primary epithelial tumours of the appendix in a predominantly black population. It also describes the rate of synchronous neoplasia and highlights the need for routine histopathology. Thus, it is of reference value to the international scientific medical community.

REFERENCES

1 **Deans GT**, Spence RA. Neoplastic lesions of the appendix. *Br J Surg* 1995; **82**: 299-306
2 **Connor SJ**, Hanna GB, Frizelle FA. Appendiceal tumors: retrospective clinicopathologic analysis of appendiceal tumors from 7,970 appendectomies. *Dis Colon Rectum* 1998; **41**: 75-80
3 **Goede AC**, Caplin ME, Winslet MC. Carcinoid tumour of the appendix. *Br J Surg* 2003; **90**: 1317-1322
4 **Jones AE**, Phillips AW, Jarvis JR, Sargen K. The value of routine histopathological examination of appendicectomy specimens. *BMC Surg* 2007; **7**: 17
5 **Modlin IM**, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 2003; **97**: 934-959
6 **Stinner B**, Rothmund M. Neuroendocrine tumours (carcinoids) of the appendix. *Best Pract Res Clin Gastroenterol* 2005; **19**: 729-738
7 **Tchana-Sato V**, Detry O, Polus M, Thiry A, Detroz B, Maweja S, Hamoir E, Defechereux T, Coimbra C, De Roover A, Meurisse M, Honoré P. Carcinoid tumor of the appendix: a consecutive series from 1237 appendectomies. *World J Gastroenterol* 2006; **12**: 6699-6701
8 **Gaskin DA**, Gaskin PS, Williams NP. Gastrointestinal carcinoids in Jamaica: 1966-2002. *West Indian Med J* 2005; **54**: 59
9 **Modlin IM**, Sandor A. An analysis of 8305 cases of carcinoid tumors. *Cancer* 1997; **79**: 813-829
10 **McGory ML**, Maggard MA, Kang H, O'Connell JB, Ko CY. Malignancies of the appendix: beyond case series reports. *Dis Colon Rectum* 2005; **48**: 2264-2271
11 **Sandor A**, Modlin IM. A retrospective analysis of 1570 appendiceal carcinoids. *Am J Gastroenterol* 1998; **93**: 422-428
12 **O'Donnell ME**, Carson J, Garstin WI. Surgical treatment of malignant carcinoid tumours of the appendix. *Int J Clin Pract* 2007; **61**: 431-437
13 **Williams GR**, du Boulay CE, Roche WR. Benign epithelial neoplasms of the appendix: classification and clinical associations. *Histopathology* 1992; **21**: 447-451
14 **Khan MN**, Moran BJ. Four percent of patients undergoing colorectal cancer surgery may have synchronous appendiceal neoplasia. *Dis Colon Rectum* 2007; **50**: 1856-1859

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Carbon dioxide for gut distension during digestive endoscopy: Technique and practice survey

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Abstract

AIM: To assess the adoption of Carbon dioxide (CO_2) insufflation by endoscopists from various European countries, and its determinants.

METHODS: A survey was distributed to 580 endoscopists attending a live course on digestive endoscopy.

RESULTS: The response rate was 24.5%. Fewer than half the respondents (66/142, 46.5%) were aware of the fact that room air can be replaced by CO_2 for gut distension during endoscopy, and 4.2% of respondents were actually using CO_2 as the insufflation agent. Endoscopists aware of the possibility of CO_2 insufflation mentioned technical difficulties in implementing the system and the absence of significant advantages of CO_2 in comparison with room air as barriers to adoption in daily practice (84% and 49% of answers, respectively; two answers were permitted for this item).

CONCLUSION: Based on this survey, adoption of CO_2 insufflation during endoscopy seems to remain relatively exceptional. A majority of endoscopists were not aware of this possibility, while others were not aware of recent technical developments that facilitate CO_2 implementation in an endoscopy suite.

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Key words: Colonoscopy; Practice survey; Carbon dioxide; Digestive endoscopy

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INTRODUCTION

Use of Carbon dioxide (CO_2) as the insufflating gas during colonoscopy was proposed in 1974 to decrease the explosion hazard associated with polypectomy[1]. As it appeared that there was less bloating and likely less pain after procedures using CO_2 for gut distension compared to air[2], randomized controlled trials (RCTs) were performed to compare post-procedure pain when using CO_2 versus room air as the insufflation agent. The results of all of these RCTs were unambiguous, with significantly less pain reported after CO_2 colonoscopy[3-7]. For other endoscopic procedures also, CO_2 was found to be superior to air: (1) for double balloon enteroscopy, small bowel intubation is deeper[8]; (2) for endoscopic retrograde cholangio-pancreatography (ERCP), post-procedural pain is less[9,10]; and (3) for complex colorectal procedures (endoscopic submucosal dissection), fewer sedative drugs are required[11]. This is explained by the pathophysiology of gases: intestinal gases leave the body through alimentary orifices and exhaled air (gases can diffuse through the gut into splanchnic blood and subsequently pulmonary circulation). Experimental studies in live animals have shown that the clearance of gas from isolated bowel segments is much faster for CO_2 than nitrogen or oxygen (the two main components of air), and this by a factor of 160 and 12, respectively[12]. The most important reason for this is the higher solubility of CO_2 compared to other gases in water. Other factors that influence the diffusion of gases through the intestinal barrier are less significant

(e.g. gas tension gradient between the intestinal lumen and blood) or identical for all digestive gases (e.g. surface and thickness of the exchange membrane, and tissue perfusion)[13].

Despite the high level of evidence supporting the use of CO_2 for gut distension during colonoscopy and other endoscopic procedures, this gas does not seem to be used in many endoscopy practices. We here report a survey that was performed in a large group of endoscopists to assess the use of CO_2 insufflation in daily endoscopy practice, including reasons for possible non-adoption.

MATERIALS AND METHODS

Survey design and administration

A questionnaire was developed by the authors for the study. Content validity of the survey was determined based on input by experts in the field and a review of the relevant literature. The final, two-page, 26-item, survey contained two parts: the first one addressed respondents' demographic characteristics and knowledge about the use of CO_2 as room air replacement during gastrointestinal endoscopy; and the second part was divided in two sections directed to endoscopists who, either did ("practitioners"), or did not ("non-practitioners") use CO_2. Non-practitioners were asked for which reasons they did not use CO_2, while practitioners were asked about their actual use of CO_2.

The survey was performed during the 26th European Workshop on Gastroenterology and Endotherapy held in Brussels, Belgium, on 16-18 June 2008. Questionnaires were placed in cases distributed to course participants, and attendees were asked to deposit completed surveys in a dedicated box at the registration desk. Consent to participate in this study was inferred from voluntary completion of the survey. Efforts to increase response rates included two rehearsals by the course director (Deviere J), projection of a reminder slide during breaks, and collection of surveys by staff members who passed between rows of participants or were posted at the exits of the projection rooms. No gift or financial incentive was proposed to attendees.

Statistical analysis

Results are expressed as mean ± SD or as a percentage. Each response was included in the analysis, regardless of the completeness of the survey. In cases when not all survey respondents answered to an individual question, the number of respondents (i.e. the denominator for percentage calculations) is indicated.

RESULTS

Study population

Surveys were distributed to 580 medical doctors attending the course, and 142 of them completed the study (response rate, 24.5%). All of them answered all the demographic questions (Table 1). The respondents had their endoscopy practice in 21 countries, but six of these (Belgium, Greece, Italy, France, Spain and Switzerland) made up two-thirds of the respondents. Main practices were roughly equally distributed between private practice, community hospitals and university hospitals. Sedation with propofol or general anesthesia was used for more than 50% of colonoscopies by about half the respondents.

Table 1 Demographic characteristics of the 142 survey respondents (mean ± SD)

Characteristics	*n* (%)
Male gender	109 (76.8)
Age (yr)	47.7 ± 9.1
Years in practice	17.5 ± 9.2
Country	
Belgium	25 (17.6)
Greece	18 (12.7)
Italy	18 (12.7)
France	16 (11.3)
Spain	10 (7.0)
Switzerland	9 (6.3)
Other	46 (32.4)
Main practice setting	
Private	36 (25.4)
Community Hospital	54 (38.0)
University Hospital	52 (36.6)
No. of colonoscopies performed/year in the center	
< 500	7 (4.9)
500-1000	40 (28.2)
1000-1500	33 (23.2)
> 1500	62 (43.7)
Proportion of colonoscopies performed with propofol/general anesthesia	
< 20%	74 (52.1)
20%-39%	4 (2.8)
40%-59%	0
60%-79%	15 (10.6)
80%-100%	49 (34.5)
Main patient pattern	
Outpatients	48 (33.8)
Inpatients	5 (3.5)
Mixed	89 (62.7)

Answers to the survey

Fewer than half of the respondents (66/142, 46.5%) were aware that room air could be replaced by CO_2 for gut distension during endoscopy. Thirty-eight respondents (26.8%) had previously seen (n = 24) or performed (n = 14) an endoscopy procedure using CO_2, with only six of them actually practicing this technique (adoption rate of the technique in the whole population, 4.2%). Fifty-eight (87.9%) of the 66 respondents who were aware of the technique also stated that all RCTs had shown that CO_2 insufflation decreased pain and gut distension compared to air insufflation. The proportions of survey respondents who correctly answered questions relating to various aspects of CO_2 use during endoscopy are shown in Figure 1.

One hundred and thirty endoscopists answered why they did not use CO_2: 73 (56.1%) of them were not aware of this possibility, and those who were aware most often cited "technical difficulties in implementing the

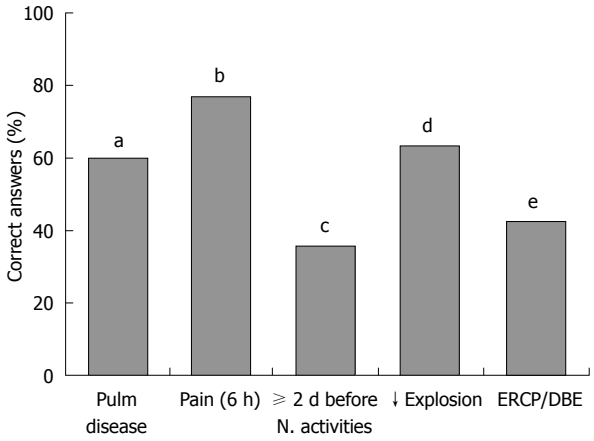

Figure 1 Percentages of correct answers (yes/no choice; correct answer was yes in all cases) to the following questions. [a]CO_2 insufflation is not advised in patients with severe pulmonary diseases; [b]About 20% of patients still have pain 6 h after colonoscopy using air insufflation; [c]About 20% of patients need ⩾ 2 d before they are able to return to their normal activities after screening colonoscopy; [d]Compared to air, CO_2 colonoscopy decreases the risk of bowel explosion; [e]Compared to air, CO_2 insufflation is better for ERCP and double balloon enteroscopy DBE.

system" and "advantages not significant enough for the patient" [n = 48 (84%) and 28 (49%), respectively; two answers were permitted for this item]. Marginal answers included the risk of patient carbonarcosis (n = 6) and of CO_2 inhalation by the endoscopy personnel (n = 4).

Reasons that could motivate a change in their practice were stated by 127 endoscopists: a demonstration of the use of CO_2 [in a workshop (n = 61; 48.0%) or in their endoscopy unit (n = 50; 39.4%)], and proposal of a CO_2 insufflator as an option when buying a colonoscope (n = 44; 34.6%) were the most frequently cited answers. Other answers were less frequent [5% higher reimbursement for CO_2 compared to air colonoscopy (n = 16; 12.6%); virtual colonoscopy performed close to their practice using CO_2 insufflation (n = 9; 7.1%)]. Four endoscopists reported that they had attempted implementing CO_2, but that they had abandoned it because of costs.

Four endoscopists who were actually using CO_2 compared it with air colonoscopy. CO_2 was rated as similar to air in terms of ease of use, endoscopist comfort and patient comfort during colonoscopy, but better with respect to post-procedure patient comfort, and more expensive (all answers were identical, except for one endoscopist who rated CO_2 as better for patient comfort during colonoscopy).

DISCUSSION

CO_2 was used for gut distension during endoscopy by < 5% of survey respondents, even though all RCTs performed since the description of the technique 35 years ago have shown that pain is lower with CO_2 compared to air[1,3-6,9,14]. Indeed, the adoption rate found in the present study was even lower than that reported 20 years ago in a survey of US hospitals in Illinois (13% for colonoscopy)[15]. A majority of endoscopists were not aware at all of the possible use of CO_2 during endoscopy, while the remainder ignored recent practical developments (they cited technical difficulties in implementing CO_2 as the main factor limiting its adoption, even though CO_2 insufflators have become more widely available). The other major reason cited for not adopting CO_2 was that advantages for the patients were not sufficiently significant. This likely relates to a lack of information among endoscopists about post-colonoscopy patient inconvenience (only one-third of them knew that 20% of patients need ⩾ 2 d before being able to return to their normal activities after screening colonoscopy)[16].

Endoscopists currently pay more attention to patients' comfort; for example, polyethylene glycol is being replaced by sodium phosphate for bowel preparation before colonoscopy[17]. However, recent reports have shown that phosphate nephropathy may complicate bowel preparation using sodium phosphate, even after a single preparation[18]. Another example is the use of propofol for sedation in replacement of benzodiazepines[19]. CO_2 deals with the post-procedure phase of colonoscopy by reducing bloating and abdominal pain, the most frequent side effects of colonoscopy[16]. However, it remains to be demonstrated if the advantages conferred by CO_2 are sufficiently significant to improve patient acceptance of endoscopic procedures and cost-effectiveness (by reducing loss from normal activities after endoscopy). These two criteria, namely patient acceptance and cost-effectiveness, are of paramount importance for colorectal cancer screening as computed tomography (CT) colonography has been shown to be superior to colonoscopy for both of them[20,21]. Incidentally, one of the three CO_2 insufflators that are available for endoscopy was developed initially for gut distension during CT colonography, and radiologists use it increasingly often for reasons of safety and patient comfort (CO_2 is used in about half of CT colonographies)[22]. In our survey, the use of CO_2 for CT colonography was not perceived by endoscopists as an incentive to change their practice. As endoscopists become aware of the ease and benefits of CO_2 implementation in an endoscopy suite, the use of CO_2 may be the next logical step to minimize patient discomfort.

Most endoscopists reported that a demonstration (in their endoscopy unit or in a workshop) was likely to change their perception of CO_2 usefulness. This corroborates our previous observation that endoscopists' opinion may significantly change following a demonstration of a particular endoscopy technique[23]. However, it remains to be seen if intentions translate into actual changes, in particular, because CO_2 benefits are mainly observed after sedation reversal, when many patients are not evaluated by endoscopists.

From a practical standpoint, CO_2 is readily available in centers where laparoscopic surgery is performed (or it can be purchased from various distributors), and endoscopic CO_2 insufflators have recently become more

Table 2 Characteristics of CO_2 insufflators available for gut distension during endoscopy

	CO_2-efficient	Olympus keymed ECR	Olympus UCR
Weight (kg)	9.0	26.0	4.9
Size (mm)	254 × 140 × 254	420 × 1049 × 539	130 × 156 × 334
Output of CO_2 adjustable	No[1]	No	No
Indicator of the amount of gas delivered	Yes	No	No
Indicator of "empty tank"	Yes	Yes	Yes
FDA approved/CE mark	Yes/Yes	Yes/Yes	Yes/Yes
Availability	International	United Kingdom	International
Price (euros)	7400	NA	7000
Manufacturer	Bracco Imaging SPA, San Donato Milanese, Italy	Olympus Keymed, Southend-on-Sea, UK	Olympus, Tokyo, Japan

FDA: Food and Drug Administration; NA: not available. [1]When the vent hole of the insufflation/irrigation valve is not occluded, CO_2 flow decreases from 3 L/min to a managed flow of 0.25 L/min, in order to preserve CO_2 reserves. None of the three models allows selecting between different intensities of CO_2 flow (in contrast with the selection of low/medium/high intensities of air flow with air insufflators).

widely available (Table 2). CO_2 insufflators are electrically powered devices that combine at the minimum a gas pressure regulator, a safety pressure valve to protect against over-insufflation, and connection tubes. When CO_2 is used, the regular air insufflation is inactivated (to prevent endoscopic insufflation with both gases), and endoscope manipulation is unchanged compared to using air for gut distension (CO_2 insufflation is obtained by placing the finger on the vent hole of the insufflation/irrigation valve, and lens cleaning is obtained by firmly pressing this valve). One may also switch from one gas to another during an endoscopic procedure. Contraindications to the use of CO_2 are limited to severe chronic obstructive pulmonary disease (if CO_2 is absorbed at a rate exceeding its respiratory elimination, this leads to CO_2 retention and pulmonary acidosis)[24]. Provided that this contraindication is observed, Bretthauer *et al*[3] have shown that, although pCO_2 levels increase during colonoscopy and ERCP (due to the effect of sedative drugs), this increase is no more important with CO_2 than with air insufflation[4,9].

Finally, the cost of an insufflator was cited as a limiting factor by endoscopists who attempted to implement the system. The cost of an insufflator ranges between 7000 and 7400 euros. The cost of CO_2 gas per colonoscopy is < 1 euro (renting a 2400-L CO_2 tank costs about 50 euros/year, and refilling it costs 25 euros; this volume is sufficient for 800 min of continuous insufflation; a mean of 8.3 L is used per colonoscopy procedure)[25]. The acquisition cost should be viewed in light of the multiple uses of these systems (e.g. colonoscopy, ERCP, double balloon enteroscopy) and ideally, from a societal perspective. Indeed, if cost calculations of screening colonoscopy took into account total time lost from work for patients undergoing the examination, as well as for the person accompanying the patient, this would increase the cost by about 50%[26]. A catalyst for CO_2 adoption by endoscopists could be the implementation of CO_2 insufflation capabilities into standard endoscopy processors, as additional costs would be hard to justify in the absence of specific reimbursement. The endoscope manufacturer that would first take this step would have a competitive advantage.

Our study has several potential limitations, including selection bias and the relatively limited number of responders. However, survey respondents were distributed relatively evenly between different endoscopy practices, and an international audit with a larger panel of individual respondents than reported here is notably difficult to organize[27,28].

In conclusion, the use of CO_2 for gut distension during endoscopy remains exceptional despite the results of numerous RCTs that have shown the superiority of this technique compared to air. A majority of endoscopists are unaware of this possibility, while those who are aware mostly think that CO_2 implementation in an endoscopy suite is technically difficult or presents few advantages. Greater availability of CO_2 insufflators, more widespread use of CO_2 in competing CT colonography, and better endoscopists' education have the potential to change this situation.

COMMENTS

Background

Carbon dioxide (CO_2) is cleared much more rapidly than air from the bowel and randomized controlled trials have consistently shown that it is superior to air for several gastrointestinal endoscopy procedures. In particular, advantages were demonstrated for colonoscopy (less pain), endoscopic retrograde cholangiopancreatography (less pain), double balloon enteroscopy (deeper bowel intubation), and long, complex, therapeutic procedures (fewer sedative drugs).

Research frontiers

Use of CO_2 is common for colon computed tomography but it does not seem to be widespread in endoscopy practice. Reasons for possible non-adoption of this gas are unknown.

Innovations and breakthroughs

No data about the use of CO_2 by endoscopists have been available for > 20 years. Recently, CO_2 insufflators for endoscopy have become commercially available.

Applications

As a majority of endoscopists were not aware of the possibility to use CO_2 as air replacement during endoscopy, specific endoscopists' education and implementation of CO_2 insufflation capabilities into standard endoscopy processors should be encouraged.

Peer review

The cost of equipment required for CO_2 insufflation during endoscopy is the main barrier to adoption of this technique; it is actually around 7000 euros.

REFERENCES

1 **Rogers BH**. The safety of carbon dioxide insufflation during colonoscopic electrosurgical polypectomy. *Gastrointest Endosc* 1974; **20**: 115-117
2 **Hussein AM**, Bartram CI, Williams CB. Carbon dioxide insufflation for more comfortable colonoscopy. *Gastrointest Endosc* 1984; **30**: 68-70
3 **Bretthauer M**, Thiis-Evensen E, Huppertz-Hauss G, Gisselsson L, Grotmol T, Skovlund E, Hoff G. NORCCAP (Norwegian colorectal cancer prevention): a randomised trial to assess the safety and efficacy of carbon dioxide versus air insufflation in colonoscopy. *Gut* 2002; **50**: 604-607
4 **Bretthauer M**, Lynge AB, Thiis-Evensen E, Hoff G, Fausa O, Aabakken L. Carbon dioxide insufflation in colonoscopy: safe and effective in sedated patients. *Endoscopy* 2005; **37**: 706-709
5 **Stevenson GW**, Wilson JA, Wilkinson J, Norman G, Goodacre RL. Pain following colonoscopy: elimination with carbon dioxide. *Gastrointest Endosc* 1992; **38**: 564-567
6 **Sumanac K**, Zealley I, Fox BM, Rawlinson J, Salena B, Marshall JK, Stevenson GW, Hunt RH. Minimizing postcolonoscopy abdominal pain by using CO(2) insufflation: a prospective, randomized, double blind, controlled trial evaluating a new commercially available CO(2) delivery system. *Gastrointest Endosc* 2002; **56**: 190-194
7 **Church J**, Delaney C. Randomized, controlled trial of carbon dioxide insufflation during colonoscopy. *Dis Colon Rectum* 2003; **46**: 322-326
8 **Domagk D**, Bretthauer M, Lenz P, Aabakken L, Ullerich H, Maaser C, Domschke W, Kucharzik T. Carbon dioxide insufflation improves intubation depth in double-balloon enteroscopy: a randomized, controlled, double-blind trial. *Endoscopy* 2007; **39**: 1064-1067
9 **Bretthauer M**, Seip B, Aasen S, Kordal M, Hoff G, Aabakken L. Carbon dioxide insufflation for more comfortable endoscopic retrograde cholangiopancreatography: a randomized, controlled, double-blind trial. *Endoscopy* 2007; **39**: 58-64
10 **Keswani R**, Hovis R, Edmunowicz S, Sadeddin E, Jonnalagadda S, Azar R, Waldbaum L, Maple J. Carbon dioxide (CO2) insufflation during ERCP for the reduction of post-procedure pain: preliminary results of a randomized, double-blind controlled trial. *Gastrointest Endosc* 2008; **67**: AB107
11 **Saito Y**, Uraoka T, Matsuda T, Emura F, Ikehara H, Mashimo Y, Kikuchi T, Kozu T, Saito D. A pilot study to assess the safety and efficacy of carbon dioxide insufflation during colorectal endoscopic submucosal dissection with the patient under conscious sedation. *Gastrointest Endosc* 2007; **65**: 537-542
12 **Mciver MA**, Redfield AC, Benedict EB. Gaseous exchange between the blood and the lumen of the stomach and intestines. *Am J Physiol* 1926; **76**: 92-111
13 **Saltzman HA**, Sieker HO. Intestinal response to changing gaseous environments: normobaric and hyperbaric observations. *Ann N Y Acad Sci* 1968; **150**: 31-39
14 **Bretthauer M**, Hoff G, Thiis-Evensen E, Grotmol T, Holmsen ST, Moritz V, Skovlund E. Carbon dioxide insufflation reduces discomfort due to flexible sigmoidoscopy in colorectal cancer screening. *Scand J Gastroenterol* 2002; **37**: 1103-1107
15 **Phaosawasdi K**, Cooley W, Wheeler J, Rice P. Carbon dioxide-insufflated colonoscopy: an ignored superior technique. *Gastrointest Endosc* 1986; **32**: 330-333
16 **Ko CW**, Riffle S, Shapiro JA, Saunders MD, Lee SD, Tung BY, Kuver R, Larson AM, Kowdley KV, Kimmey MB. Incidence of minor complications and time lost from normal activities after screening or surveillance colonoscopy. *Gastrointest Endosc* 2007; **65**: 648-656
17 **Tan JJ**, Tjandra JJ. Which is the optimal bowel preparation for colonoscopy - a meta-analysis. *Colorectal Dis* 2006; **8**: 247-258
18 **Heher EC**, Thier SO, Rennke H, Humphreys BD. Adverse renal and metabolic effects associated with oral sodium phosphate bowel preparation. *Clin J Am Soc Nephrol* 2008; **3**: 1494-1503
19 **Rex DK**, Heuss LT, Walker JA, Qi R. Trained registered nurses/endoscopy teams can administer propofol safely for endoscopy. *Gastroenterology* 2005; **129**: 1384-1391
20 **Hassan C**, Pickhardt PJ, Laghi A, Kim DH, Zullo A, Iafrate F, Di Giulio L, Morini S. Computed tomographic colonography to screen for colorectal cancer, extracolonic cancer, and aortic aneurysm: model simulation with cost-effectiveness analysis. *Arch Intern Med* 2008; **168**: 696-705
21 **Gluecker TM**, Johnson CD, Harmsen WS, Offord KP, Harris AM, Wilson LA, Ahlquist DA. Colorectal cancer screening with CT colonography, colonoscopy, and double-contrast barium enema examination: prospective assessment of patient perceptions and preferences. *Radiology* 2003; **227**: 378-384
22 **Pickhardt P**. Incidence of significant complications at CT colonography: collective experience of the working group on virtual colonoscopy. *Gastrointest Endosc* 2006; **63**: AB202
23 **Dumonceau JM**, Dumortier J, Deviere J, Kahaleh M, Ponchon T, Maffei M, Costamagna G. Transnasal OGD: practice survey and impact of a live video retransmission. *Dig Liver Dis* 2008; **40**: 776-783
24 **Nguyen NT**, Wolfe BM. The physiologic effects of pneumoperitoneum in the morbidly obese. *Ann Surg* 2005; **241**: 219-226
25 **Bretthauer M**, Hoff GS, Thiis-Evensen E, Huppertz-Hauss G, Skovlund E. Air and carbon dioxide volumes insufflated during colonoscopy. *Gastrointest Endosc* 2003; **58**: 203-206
26 **Jonas DE**, Russell LB, Sandler RS, Chou J, Pignone M. Value of patient time invested in the colonoscopy screening process: time requirements for colonoscopy study. *Med Decis Making* 2008; **28**: 56-65
27 **Goel A**, Barnes CJ, Osman H, Verma A. National survey of anticoagulation policy in endoscopy. *Eur J Gastroenterol Hepatol* 2007; **19**: 51-56
28 **Ladas SD**, Aabakken L, Rey JF, Nowak A, Zakaria S, Adamonis K, Amrani N, Bergman JJ, Boix Valverde J, Boyacioglu S, Cremers I, Crowe J, Deprez P, Díte P, Eisen M, Eliakim R, Fedorov ED, Galkova Z, Gyokeres T, Heuss LT, Husic-Selimovic A, Khediri F, Kuznetsov K, Marek T, Munoz-Navas M, Napoleon B, Niemela S, Pascu O, Perisic N, Pulanic R, Ricci E, Schreiber F, Svendsen LB, Sweidan W, Sylvan A, Teague R, Tryfonos M, Urbain D, Weber J, Zavoral M. Use of sedation for routine diagnostic upper gastrointestinal endoscopy: a European Society of Gastrointestinal Endoscopy Survey of National Endoscopy Society Members. *Digestion* 2006; **74**: 69-77

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BRIEF ARTICLES

Positional effect of mutations in 5'UTR of hepatitis C virus 4a on patients' response to therapy

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Abstract

AIM: To investigate the effects of mutations in domain Ⅲ of the hepatitis C virus (HCV) internal ribosome entry sequences (IRES) on the response of chronic HCV genotype 4a patients to interferon therapy.

METHODS: HCV RNA was extracted from 19 chronic HCV 4a patients receiving interferon/ribavirin therapy who showed dramatic differences in their response to combination therapy after initial viral clearance. IRES domain Ⅲ was cloned and 15 clones for each patient were sequenced. The obtained sequences were aligned with genotype 4a prototype using the ClustalW program and mutations scored. Prediction of stem-loop secondary structure and thermodynamic stability of the major quasispecies in each patient was performed using the MFOLD 3.2 program with Turner energies and selected constraints on base pairing.

RESULTS: Analysis of RNA secondary structure revealed that insertions in domain Ⅲ altered Watson-Crick base pairing of stems and reduced molecular stability of RNA, which may ultimately reduce binding affinity to ribosomal proteins. Insertion mutations in domain Ⅲ were statistically more prevalent in sustained viral response patients (SVR, n = 14) as compared to breakthrough (BT, n = 5) patients.

CONCLUSION: The influence of mutations within domain Ⅲ on the response of HCV patients to combination therapy depends primarily on the position, but not the frequency, of these mutations within IRES domain Ⅲ.

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Key words: Hepatitis C virus; Internal ribosome entry sequences; Domain Ⅲ; Genotype 4a; Ribosomal subunit; Interferon therapy; RNA folding

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INTRODUCTION

The hepatitis C virus (HCV) genome is a 9.5 Kb single stranded RNA molecule of positive polarity. It contains a single open reading frame flanked by 5' and 3' untranslated regions (UTR) of 341 and about 230 nucleotides, respectively[1]. Both the 5' and 3' UTRs contain conserved RNA structures essential for polyprotein translation and genome replication[2]. The 5'UTR is uncapped and contains the internal ribosomal

entry site (IRES) which contains highly conserved secondary and tertiary structures that are essential for proper binding and positioning of the viral RNA within the host cell's protein translation machinery[3,4].

The only FDA approved medication for the treatment of HCV infection is the combination therapy of either standard or pegylated interferon and ribavirin[5]. However, only 28%-60% of HCV patients respond to the treatment, depending on the viral genotype[6,7]. Resistance to interferon therapy is believed to be controlled by host and viral factors[8]; a significant viral factor is the generation of different HCV quasispecies[9]. The critical role of 5'UTR in initiation of polyprotein translation requires the highest degree of conservation of this region. It contains four highly structured domains numbered Ⅰ to Ⅳ; all are required for recruiting, positioning, and activating/regulating the host protein synthesis machinery[10]. Translation initiation in HCV starts by binding of the HCV IRES to the 40S subunit and assembly of this binary complex with eukaryotic initiation factor 3 (eIF3). The IRES-40S-eIF3 ternary complex combines with eIF2/GTP/initiator tRNA forming the 48S complex. Progression from 48S to 80S initiation complexes is a slow step during IRES-mediated initiation which may reflect a decrease or absence of factor activity or conformational rearrangements in the IRES leading to sub-unit joining[11]. The need for flexible rather than rigid binding allows the conformational rearrangement and subsequent efficient sub-unit joining leading to initiation of translation. Therefore low affinity binding of factors due to mutations in binding sites may be associated with more flexibility and efficient initiation of translation.

The 5'UTR can be divided into the 5' and 3' parts. The 5' part is near to a fully single stranded structure and includes domain Ⅰ. The 3' part is highly structured and is essential for HCV IRES function. It folds into three additional domains Ⅱ, Ⅲ and Ⅳ. Domain Ⅱ is a stem with several internal loops. Domain Ⅳ is a small hairpin which includes the AUG start codon. The pseudo knot joins domain Ⅱ with domain Ⅲ and is base-paired to the sequence directly upstream of domain Ⅳ.

The HCV IRES forms an extended structure that binds the 40S subunit by several synergetic interactions, and the domains involved in this binding have been determined by chemical and enzymatic foot-printing experiments[12]. The segment starting from nucleotide 141 to 279 which compromises domain Ⅲ, the focus of the present study, is highly conserved at the primary nucleotide sequence as well as at secondary and tertiary structure levels. The basal part of domain Ⅲ (including principally the pseudo knot and stem-loop Ⅲd) includes the elements of secondary structure that determine the binding of the IRES to the 40S subunit. Besides providing affinity for the 40S subunit, the basal part of domain Ⅲ is thought to be required for correct positioning of the initiation codon in the decoding center of the 40S subunit, as indicated by "toe-printing" experiments[11]. The apical part of domain Ⅲ binds eIF3 *via* stem-loop Ⅲb and the four-way junction and is required for 40S and eIF3 binding.

In the present study, the positional effect of mutations on the predicted secondary structure and thermodynamic stability of domain Ⅲ was examined in 19 chronic HCV type 4a patients with initial virological response defined as undetectable viremia after 12 wk from start of treatment. Only 14 patients achieved sustained virological response (SVR) i.e. negative viremia 24 wk after end of treatment. In the remaining 5 patients who suffered virological breakthrough (BT), the predicted stem-loop structures and thermodynamic stability of domain Ⅲ were compared in both pre- and post-treatment samples.

MATERIALS AND METHODS

HCV patients

Male or female patients (n =19; 18-60 years) with chronic active hepatitis C virus infection were included in this study. Patients were negative for HBsAg and HBsAb but positive for anti-HCV and HCV-RNA by RT- PCR. All had elevated ALT and AST levels and received combined therapy of pegylated IFNα-2b (100 μg/wk) plus ribavirin (800-1000 mg/d). Patients had normal values for blood counts, other liver functions, auto immune markers, T3, T4 and TSH, renal functions, blood sugar and α-fetoprotein. None of the patients had other causes of liver disease (e.g. α1 antitrypsin deficiency, Wilson's disease, alcoholic or decompensated liver disease, obesity-induced liver disease, drug-related liver disease), no CNS trauma, or active seizures, no ischemic cardiovascular disease within the last six months or hemochromatosis. None was co-infected with HBV or schistosomiasis.

Cloning and sequencing of the HCV domain Ⅲ

RNA extraction and reverse transcription-PCR of HCV RNA, using Qiagen single step RT-PCR kit (Qiagen, Inc., Chatsworth, CA, USA), were performed as described previously[13,14]. Amplification of 266 bp was performed in a single step using primer pair; forward (nt 47-68) 5'-GTGAGGAACTACTGTCTTCACG-3' and reverse (nt 292-312) 5'-ACTCGCAAGCACCCTATC AGG-3'. Cloning of amplified products was done with TA cloning kit (Invitrogen Co., Carlsbad, CA). Fifteen clones from each subject were sequenced using the TRUGENE HCV 5-NC genotyping kit, Visible Genetics, Inc. (Toronto, Ontario, Canada) in conjunction with the Open Gene DNA sequencing system. The insert DNA was sequenced by CLIP sequencing which allows both directions of the target amplicon to be sequenced simultaneously in the same tube using two different dye labeled primers (Cy5.0 and Cy5.5) for each reaction. This method provides sequence information for both positive and negative DNA strands from a single reaction. The obtained sequences were then aligned with genotype 4a prototype using the ClustalW program (www.ch.embnet.org/software/ClustalW.html). Among the 15 domain Ⅲ sequences obtained for each patient only one sequence was found in the majority of the clones, thus representing the major quasispecies of domain Ⅲ in each patient.

Table 1 Distribution of mutations in different loops of domain Ⅲ in SVR compared with BT patients

Mutation	Position	Region	No. of patients			Effect on ΔG (kcal/mol)
			SVR (n = 14)	BT-PreT (n = 5)	BT Post-T (n = 5)	
C-T	148	Junction joining stem-loop Ⅲa b, c & d	-	-	1	Increased
G-T	158	Loop Ⅲa	-	1	4	Increased
T-A	175	Loop Ⅲb	1	-	-	No effect
G & A ins	179		2	-	-	Increased
T ins.	180		1	-	-	Increased
T ins.	181		1	-	-	Increased
C-T	186		-	-	1	No effect
TTT-GGG	194-196		-	1	-	No effect
C ins.	205		1	-	-	No effect
T-C	199		-	-	1	No effect
G-A	243	Junction joining stem-loop Ⅲa, b, c & d	5	1	4	No effect
C-T	254	Loop Ⅲd	-	1	1	Decreased
A-C	260		1	-	1	Decreased
A-T	260		1	-	-	Decreased
G-T	261		1	-	-	Increased
G-T	268		-	1	1	Decreased
T-C	269		-	1	1	Decreased
G ins.	270		-	1	1	Decreased
A-C	275		2	-	-	Decreased

The distribution of the observed mutations in different loops of domain Ⅲ in sustained viral response (SVR) and breakthrough (BT) patients is shown. The majority of mutations (14/19) were simple substitutions whereas 5/19 were insertions. Most mutations were found in stem-loops Ⅲb and d. The effect of each mutation on the overall thermodynamic stability of the related domain is indicated. Decreased ΔG means increased stability and vice versa. Pre-T: Pre-treatment; Post-T: Post-treatment.

Sequence diversities in each major quasispecies were compared with genotype 4a and various mutation types were scored.

RNA secondary structure and thermodynamic stability

Prediction of stem-loop structure and thermodynamic stability of the major quasispecies in each patient was performed using the MFOLD 3.2 program with Turner energies and selected constraints on base pairing as indicated[15]. The program was run on EFN server: 1996-2008, Michael Zuker, Rensselaer Polytechnic Institute (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi).

RESULTS

HCV Quasispecies and genotyping

The mean number of domain Ⅲ quasispecies in pre-treatment samples of SVR and BT states was about 2 quasispecies per patient with no significant difference between both groups. The quasispecies complexity of domain Ⅲ tripled in post-treatment samples of BT patients. All patients included in the study were genotyped as 4a except for one SVR patient infected with a hybrid 4a/1b genotype.

Domain Ⅲ sequence diversities in patient study groups

Different types of mutations were compared in both study groups with the prototype sequence of HCV 4a (Table 1). Insertion mutations in domain Ⅲ were statistically more prevalent in SVR than BT patients. This is expected since insertions are known to dramatically alter the stem-loop structure by changing the Watson-Crick base pairing of stems, thus reducing molecular stability of the RNA or binding affinity to ribosomal proteins. The percentage of transition mutations was statistically higher in pre-treatment samples of BT than SVR. There were no statistical differences in percentage of transversion mutations between the two patient groups.

Distribution of mutations in different loops of domain Ⅲ in SVR compared with BT patients

Alignment of domain Ⅲ sequences derived from study patients revealed the presence of 19 point mutations scattered in stem-loops Ⅲa, b, c and d (Table 1). Percentage distribution of the identified mutations in different loops of domain Ⅲ in SVR and BT patients is presented in Table 2. The majority of mutations, 14/19 (73.7 %), were simple substitutions while 5 (26.3%) were insertions. Substitutions included 42.8% transitions and 57.2% transversions. Most of the mutations (84.2%) were localized in 2 stem-loops; Ⅲb (nt 175-205) and Ⅲd (nt 254-275), with 42.1% of the mutations in each loop. The remaining 15.8% of mutations were located in junction Ⅲa, b, c (10.5%) and in loop Ⅲa (5.3%). The SVR patients contain specific mutations (Figure 1) that were not detected in either pre-treatment BT groups or post-treatment BT patients (Table 1). These SVR specific mutations comprise 42.1% (8/19) of the total number of mutations detected in the studied patient population (Table 1). Interestingly, approximately two thirds of SVR (5/8) specific mutations were located in loop Ⅲb and one third (3/8) in Ⅲd with only one exceptional mutational event (nt 243 in junction joining loops Ⅲa, b, c, d) that was detected outside loops Ⅲb and Ⅲd. These two stem-loop structures play critical roles in directing

Table 2 Distribution of the 19 identified mutations in different loops of domain Ⅲ in SVR and BT patients

Region	Responder (SVR; %)	BT pre treatment (%)	BT post treatment (%)
Stem-loop Ⅲa (156-171)	-	14.3	10
Stem-loop Ⅲb (172-227)	50	14.3	20
Stem-loop Ⅲc (228-238)	-	-	-
Stem-loop Ⅲd (253-279)	40	57.1	50
Junction joining stem-loop Ⅲa, b, c, & d (141-153) & (239-252)	10	14.3	20

Ten mutations, out of the 19 identified point mutations, are found in responders (SVR). Five of these mutations are found in loop Ⅲb, four in Ⅲd, and one in the junction. Seven mutations are found in the BT pre-treatment group (four in loop Ⅲd and one in each of loop Ⅲa, Ⅲb, and junction). Ten mutations were detected in BT post-treatment (five in IIId, 2 in each of Ⅲb and the junction, and one in loop Ⅲa).

viral protein translation *via* recruiting and regulating ribosomal subunit proteins and cellular initiation factors for accurate positioning of HCV RNA in the translational machinery of the host cell. On the other hand, mutational events in pre-treatment samples of the BT patients were detected in almost all loop structures of domain Ⅲ, mostly located in loop Ⅲd. Most notably loop Ⅲd mutations were associated with decreased ΔG value (Table 1) indicating increased thermodynamic stability of the RNA structure, thus explaining resistance to therapy in the BT patient group. It is noteworthy that most of loop Ⅲb mutations in SVR were associated with increased ΔG value, indicating decreased thermodynamic stability of the viral RNA, therefore contributing to the multifactorial eradication of viral RNA in the SVR patient group. When comparing the mutational events in pre-treatment BT patients with those observed post-treatment, the number of mutational events known to induce significant elevation in thermodynamic stability (i.e. decreased ΔG in loop Ⅲd) did not increase after treatment in BT patients. These results suggest that domain Ⅲ-associated factors of viral breakthrough are determined by genetic events in the HCV genome before start of treatment rather than being acquired as a result of stress induced by IFNα therapy.

Prediction of thermodynamic stability and effect of mutations on the secondary structure

Changes in the minimum free energy (ΔG) were detected for stem-loops Ⅲa, b, c and d spanning nucleotides 141 to 282. Predictive stability values were compared for genotype 1b (strain H77), genotype 4a (I.D. Y11604.1) and study samples derived from SVR and BT patients. The ΔG value of genotype 1b was -53.6 kcal/mol; more stable than genotype 4a (ΔG -47.0 kcal/mol) as illustrated in the secondary structure/thermodynamic stability prediction (Figures 2 and 3). As shown in Table 1, 31.5% of the mutations have no effect on the ΔG value, 31.5% of the mutations were associated with increased ΔG i.e. reduced stability of the secondary structure, while 37% of the mutations were associated with decreased ΔG value

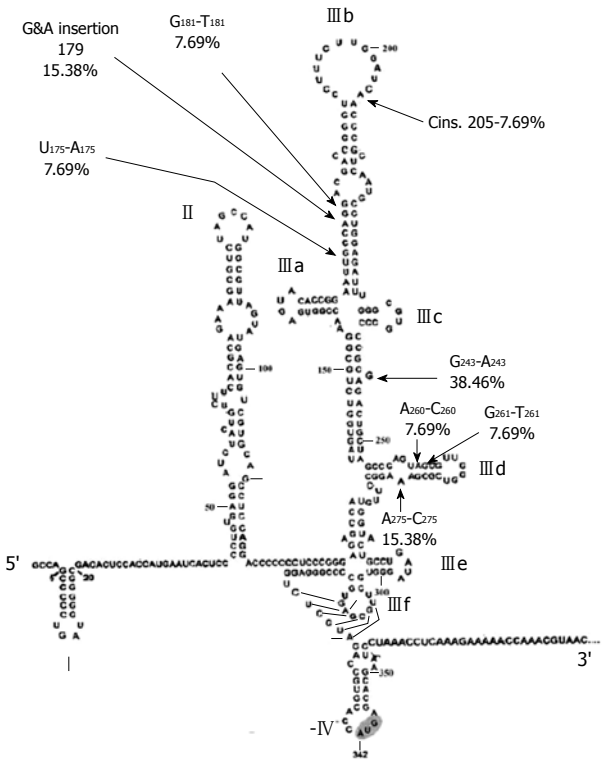

Figure 1 Predicted secondary and tertiary structure of HCV IRES[23] with mutations observed in SVR patients shown.

i.e. increased stability of the RNA secondary structure. An interesting observation (Table 1 and Figure 1) is that mutations with no effect on ΔG values were located in the apical part of domain Ⅲ secondary structure (nt 186-243), spanning mostly the upper part of loop Ⅲb involved in eIF3 binding. On the other hand, those mutations affecting the stability parameters were localized in the basal part of domain Ⅲ encompassing loops Ⅲa/b and Ⅲd. Most notably, Ⅲa/b (nt 158-199) mutations were associated with reduced stability and Ⅲd (nt 254-275) mutations were associated with increased stability of the RNA structure. Taken together, the data relating to minimum free energy in SVR and BT patients indicate that the levels of thermodynamic stability are not sufficient parameters to predict response to IFNα treatment and suggest that other parameters involving affinity of RNA binding to ribosomal subunits play important roles in determining response to treatment.

DISCUSSION

The pathway of HCV IRES-mediated initiation of translation is primarily dependent on RNA binding to several cellular proteins, of which ribosomal 40S subunit and eIF3 play pivotal roles in efficient translation of a polyprotein precursor[16]. Several foot-printing, toe-printing and UV cross linking assays have shown that domain Ⅲ is the most active part in RNA-protein binding where it folds to form stem-loop structures for high affinity binding to host proteins[11,17-19]. Although genomic variability in HCV IRES was shown as one mechanism for escaping IFNα effects, there has been

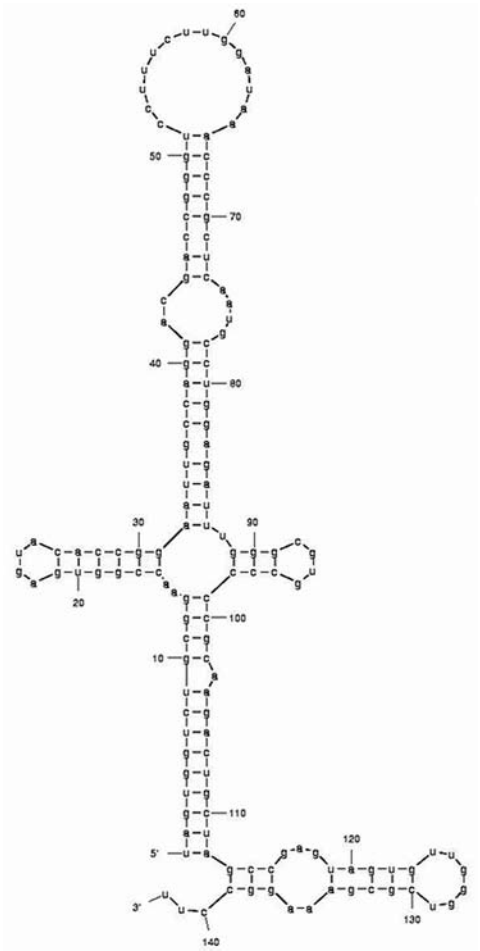

Figure 2 RNA secondary structure and thermodynamic stability of loops Ⅲa, b, c, & d from genotype 1b. The minimum free energy value (ΔG) was -53.6 kcal/mol.

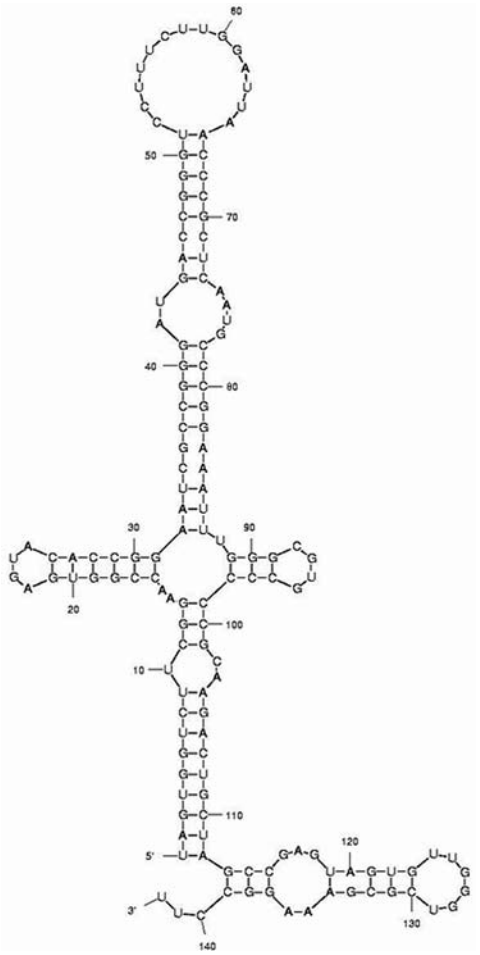

Figure 3 RNA secondary structure and thermodynamic stability of loops Ⅲa, b, c, & d from genotype 4a (prototype). The minimum free energy value (ΔG) was -47.6 kcal/mol.

controversy with regard to the predictive value of pre-treatment IRES genomic variations in determining later response to IFNα[20]. Although domain Ⅲ harbors only 22% of the overall IRES mutations in a mixed genotype population[21], IRES activity seems to depend more on the location of mutant nucleotides which play the most important roles in IRES activity.

We performed a focused study on domain Ⅲ derived from patients infected exclusively with genotype 4a and presenting a dramatic difference in sustained response after initial viral disappearance. This approach allowed us to minimize quasispecies complexity (mean of 2 variants/patient) compared with 7 or more variants/patient in other studies[21] and to pinpoint a number of important genomic determinants of response to IFNα. Cloning of domain Ⅲ and sequencing of 15 clones in each patient allowed us to identify the major variant (identical sequences in 12 clones or more) in each patient so that noise was reduced during extrapolation of the relationship between genomic variation and treatment outcome.

Disruption of base pairing in stem structure significantly inhibits IRES-dependent translation[22]. In our patient population insertion mutations in domain Ⅲ were statistically higher in SVR than in BT patients. These results are expected since insertions are known to dramatically alter the stem-loop structure by changing the Watson-Crick base pairing of stems, thus reducing molecular stability of the RNA or binding affinity to ribosomal proteins. The distributions of domain Ⅲ mutations in SVR patients were distinct from those of the BT group in the present study. Of the total number of mutations, approximately 42% were exclusive to SVR patients; almost all were located in loops Ⅲb and Ⅲd. These two stem-loop structures were implicated in initiation complex recruitment, positioning and regulation, where Ⅲd forms the core of high affinity binding with the 40S subunit[22] and recruits ribosomal elements involved in positioning of mRNA and tRNA[10]. Besides binding to eIF3, stem-loop Ⅲb interacts with a multisubunit initiation factor involved in subunit assembly and stability of ternary complex[22].

This explanation cannot simply be taken to resolve mysteries of HCV IRES in light of the apparent controversy in loop Ⅲd genomic diversity. All of Ⅲd mutations detected exclusively in BT patients (pre- and post-treatment) were associated with increased thermodynamic stability, thus leading to viral persistence;

also most of IIId mutations detected in SVR patients were again associated with increased stability of this RNA. The slow rate of ribosomal subunit transition compared to canonical translation[11] directs the attention towards the need for flexible rather than rigid binding which allows the conformational rearrangement and subsequent efficient subunit joining, leading to initiation of translation. Therefore mutations affecting binding affinity of factors regardless of thermodynamic stability of RNA structure may be associated with either more flexibility or rigidity which in turn regulates efficiency of translational initiation[11]. Taken together, the data regarding minimum free energy in SVR and BT patients indicate that the levels of thermodynamic stability are not sufficient parameters to predict response to IFNα treatment and suggest that other parameters involving affinity of RNA binding to ribosomal subunits play significant roles in determining response to treatment. The concept that mutations in BT patients appear only in post-treatment samples and are associated with no effect on RNA stability suggests that viral breakthrough is determined by mutations in domain III before start of treatment rather than being acquired during treatment. Alternatively, the roles of these mutations in viral persistence could be related to fine tuning of the flexibility of RNA structure for binding to cellular factors regardless of its stability. The former view is more plausible since the majority of IIId mutations in pre-treatment were associated with increased RNA stability without change in the frequency of mutations post-treatment. An interesting observation in this study is that the A243G mutation in the IIIc/IIId junction was detected both in SVR (5 times) and in pre-treatment BT (2 times) and was more detectable in post-treatment BT patients (4 times). Predictive folding however, revealed no effect of this mutation on the calculated thermodynamic stability. The role of nucleotide 243 in maintaining IRES structure was reported in HCV genotype 1b[21] and changes at this position were encountered in patients with viral stabilization. In genotype 1b, A243 pairs with U149, which is lacking in 4a, leading to altered pairing and explains the high rate of mutations at this position in our study population regardless of response to IFNα, thus making it more vulnerable to mutational event in genotype 4a.

In conclusion, the RNA structure of domain III in HCV IRES contains several important elements implicated in determining the response to IFNα treatment. The results presented herein demonstrate that domain III structure in SVR patients is different from BT patients. Thermodynamic stability of RNA secondary structure is a significant but not sufficient parameter for prediction of viral stabilization, or response to IFNα. Elements of binding to ribosomal subunit complexes require further studies to unravel the exact role of IRES in HCV stabilization and persistence.

COMMENTS

Background

The hepatitis C virus (HCV) is a major public health problem with about 200 million individuals currently infected with the virus (about 3% of the world's population). So far, 11 genotypes and more than 70 subtypes have been identified. The only approved FDA treatment for chronic HCV infection is the combination therapy of pegylated interferon and ribavarin. The variable response of patients to therapy ranges between 28% and 60% and has been proposed to be affected by various host and viral factors. Investigating the effect of mutations within the HCV 5'UTR, the most conserved region in the viral genome, on response to therapy is important because this region is vital for initiation of viral polyprotein translation and the ability of HCV to replicate.

Research frontiers

Although interferon and ribavirin are the only FDA approved drugs to treat HCV, they suffer from several drawbacks including severe side effects (including hematological abnormalities and neuropsychiatric symptoms), very high cost, and most importantly low therapeutic response. Consequently, factors that affect the response of HCV patients to therapy have to be addressed and results could be used for predicting response to therapy before initiation of treatment. Moreover, identification of viral factors that correlate with therapeutic response would contribute to other studies on viral and host factors. This could result in a global view and comprehensive understanding of how host and viral factors affect a patient's response to therapy.

Innovations and breakthroughs

Recent reports, using clinical specimens or HCV replicon systems from different genotypes, have highlighted the effect of mutations in different domains of the viral genome (in particular the HCV 5'UTR) on patients' response to therapy. However, limited studies have been done on genotype 4a. In the present article, The authors focused mainly on genotype 4a which is the predominant genotype in Egypt; found in over 90% of all HCV-infected patients. They showed that the thermodynamic stability of the HCV 5'UTR region is different among responders (sustained viral clearance) and breakthrough patients (who suffer relapse at the end of treatment). Additionally, their results indicate that response to therapy is related mainly to the position of mutations but not their frequency. Finally, thermodynamic stability of IRES was shown to have a direct influence on the binding of the viral genome to the host proteins, which results in initiation of the translation of the viral polyprotein.

Applications

The results of this study suggest that the presence of single nucleotide polymorphisms (SNPs) in certain positions had direct effect on the response of HCV patients to interferon therapy. Taking into consideration the positions of these mutations, different real-time PCR or other assays can be developed for detection of the SNPs to allow the prediction of the response to interferon therapy as a step for identification of patients who are more likely to respond to therapy.

Terminology

5'UTR: non-coding region of HCV RNA; contains the internal ribosomal entry site (IRES); site of initiation of translation. IRES: (internal ribosomal entry site): a structure within the HCV RNA 5'UTR that binds directly to the ribosome to initiate translation. Cap-dependent translation: the mechanism of translation (protein synthesis) predominantly used for cellular proteins. Cap-independent translation: translation *via* an internal ribosomal entry site (IRES); the mechanism utilized by HCV. Eukaryotic initiation factors: cellular proteins involved in translation. SVR: sustained viral response; patients who show negative HCV PCR results after termination of therapy.

Peer review

The authors present a sequence analysis study of HCV genotype 4a patients undergoing combination therapy. This study is of a substantial potential interest. In this work the authors propose that insertion mutations in domain III of the IRES region are more prevalent in sustained viral response patients compared with breakthrough patients and that such mutations may affect the ability of HCV virus to replicate by decreasing the thermodynamic stability of its RNA.

REFERENCES

1 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362

2 **Penin F**, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5-19

3 **Honda M**, Beard MR, Ping LH, Lemon SM. A phylogenetically conserved stem-loop structure at the 5' border of the internal ribosome entry site of hepatitis C

virus is required for cap-independent viral translation. *J Virol* 1999; **73**: 1165-1174

4 **Kieft JS**, Zhou K, Grech A, Jubin R, Doudna JA. Crystal structure of an RNA tertiary domain essential to HCV IRES-mediated translation initiation. *Nat Struct Biol* 2002; **9**: 370-374

5 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

6 **Booth JC**, O'Grady J, Neuberger J. Clinical guidelines on the management of hepatitis C. *Gut* 2001; **49** Suppl 1: I1-21

7 **Hofmann WP**, Zeuzem S, Sarrazin C. Hepatitis C virus-related resistance mechanisms to interferon alpha-based antiviral therapy. *J Clin Virol* 2005; **32**: 86-91

8 **Yeh SH**, Chen DS, Chen PJ. A prospect for pharmacogenomics in the interferon therapy of chronic viral hepatitis. *J Antimicrob Chemother* 2003; **52**: 149-151

9 **Salmeron J**, Casado J, Rueda PM, Lafuente V, Diago M, Romero-Gomez M, Palacios A, Leon J, Gila A, Quiles R, Rodriguez L, Ruiz-Extremera A. Quasispecies as predictive factor of rapid, early and sustained virological responses in chronic hepatitis C, genotype 1, treated with peginterferon-ribavirin. *J Clin Virol* 2008; **41**: 264-269

10 **Ji H**, Fraser CS, Yu Y, Leary J, Doudna JA. Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. *Proc Natl Acad Sci USA* 2004; **101**: 16990-16995

11 **Otto GA**, Puglisi JD. The pathway of HCV IRES-mediated translation initiation. *Cell* 2004; **119**: 369-380

12 **Kieft JS**, Zhou K, Jubin R, Doudna JA. Mechanism of ribosome recruitment by hepatitis C IRES RNA. *RNA* 2001; **7**: 194-206

13 **Boom R**, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; **28**: 495-503

14 **Zekri AR**, El-Din HM, Bahnassy AA, Khaled MM, Omar A, Fouad I, El-Hefnewi M, Thakeb F, El-Awady M. Genetic distance and heterogenecity between quasispecies is a critical predictor to IFN response in Egyptian patients with HCV genotype-4. *Virol J* 2007; **4**: 16

15 **Zuker M**. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003; **31**: 3406-3415

16 **Dasgupta A**, Das S, Izumi R, Venkatesan A, Barat B. Targeting internal ribosome entry site (IRES)-mediated translation to block hepatitis C and other RNA viruses. *FEMS Microbiol Lett* 2004; **234**: 189-199

17 **Sizova DV**, Kolupaeva VG, Pestova TV, Shatsky IN, Hellen CU. Specific interaction of eukaryotic translation initiation factor 3 with the 5' nontranslated regions of hepatitis C virus and classical swine fever virus RNAs. *J Virol* 1998; **72**: 4775-4782

18 **Pestova TV**, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CU. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev* 1998; **12**: 67-83

19 **Buratti E**, Tisminetzky S, Zotti M, Baralle FE. Functional analysis of the interaction between HCV 5'UTR and putative subunits of eukaryotic translation initiation factor eIF3. *Nucleic Acids Res* 1998; **26**: 3179-3187

20 **Yamamoto C**, Enomoto N, Kurosaki M, Yu SH, Tazawa J, Izumi N, Marumo F, Sato C. Nucleotide sequence variations in the internal ribosome entry site of hepatitis C virus-1b: no association with efficacy of interferon therapy or serum HCV-RNA levels. *Hepatology* 1997; **26**: 1616-1620

21 **Thelu MA**, Leroy V, Ramzan M, Dufeu-Duchesne T, Marche P, Zarski JP. IRES complexity before IFN-alpha treatment and evolution of the viral load at the early stage of treatment in peripheral blood mononuclear cells from chronic hepatitis C patients. *J Med Virol* 2007; **79**: 242-253

22 **Boehringer D**, Thermann R, Ostareck-Lederer A, Lewis JD, Stark H. Structure of the hepatitis C virus IRES bound to the human 80S ribosome: remodeling of the HCV IRES. *Structure* 2005; **13**: 1695-1706

23 **Honda M**, Brown EA, Lemon SM. Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* 1996; **2**: 955-968

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Extracolonic findings of computed tomographic colonography in Koreans

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Abstract

AIM: To determine the frequency and characteristics of extracolonic lesions detected using computed tomographic (CT) colonography.

METHODS: The significance of extracolonic lesions was classified as high, intermediate, or low. Medical records were reviewed to establish whether further investigations were carried out pertaining to the extracolonic lesions that were detected by CT colonography.

RESULTS: A total of 920 cases from 7 university hospitals were included, and 692 extracolonic findings were found in 532 (57.8%) patients. Of 692 extracolonic findings, 60 lesions (8.7%) were highly significant, 250 (36.1%) were of intermediate significance, and 382 (55.2%) were of low significance. CT colonography revealed fewer extracolonic findings in subjects who were without symptoms ($P < 0.001$), younger ($P < 0.001$), or who underwent CT colonography with no contrast enhancement ($P = 0.005$). CT colonography with contrast enhancement showed higher cost-effectiveness in detecting highly significant extracolonic lesions in older subjects and in subjects with symptoms.

CONCLUSION: Most of the extracolonic findings detected using CT colonography were of less significant lesions. The role of CT colonography would be optimized if this procedure was performed with contrast enhancement in symptomatic older subjects.

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Key words: Computed tomographic colonography; Extracolonic lesion; Cost; Contrast enhancement; Clinical availability

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INTRODUCTION

Computed tomographic (CT) colonography allows the visualization of extracolonic organs, thereby permitting

the detection of potentially significant pathologies beyond the colon[1]. Extracolonic lesions are found in 15%-85% of cases, with some being important lesions, such as extracolonic cancer or aortic aneurysm[2,3]. However, most of the extracolonic lesions are of minimal importance and lead to further investigations and possibly procedures, with the final diagnosis being simple benign pathology[2]. Thus, the evaluation and management of extracolonic findings have been found to lead to significant additional cost, and the feasibility and cost-effectiveness of CT colonography needs to be carefully evaluated[4].

Since multi-section helical CT colonography was first introduced in 1998, improvements such as faster scanning, improved temporal resolution and reduced motion artifacts have been implemented[5]. However, multidetector CT colonography has been described as a sort of Pandora's box, releasing a cascade of diagnostic events with medicolegal, ethical and economic implications[6]. Therefore, it would be helpful to clinicians if there were defined strategies for the clinical approach toward the detection of highly significant extracolonic lesions.

To the best of our knowledge, there has been no large multicenter study on extracolonic findings of CT colonography among Koreans. We therefore performed a multicenter study to assess the frequency and characteristics of extracolonic lesions detected with the aid of CT colonography. In addition, we surveyed the factors related to the detection of highly significant extracolonic findings, and analyzed its cost-effectiveness to determine which factors would enhance the potential benefits of CT colonography examination.

MATERIALS AND METHODS

Subjects

The results of CT colonographies performed from January 2005 to December 2006 at the authors' seven university hospitals in Korea were reviewed. Those who were diagnosed as having a malignancy at the time of the CT colonography, those under the age of 16 years and those with ethnicity other than Korean were excluded from the study.

Types and scanning parameters of multidetector array CT colonography are summarized in Table 1. The subjects underwent standard bowel preparation, and a rectal catheter was inserted. Air was used to distend the colon to maximum subject tolerance. Scout image was taken to confirm the adequacy of distention before each examination. Images were taken from the diaphragm to the symphysis with the subject in the supine and prone positions during a breath hold. Medical records were reviewed to establish whether further investigation was carried out pertaining to the extracolonic lesions that were detected by CT colonography during 1 year follow up period. This retrospective study was approved by the institutional review boards which confirmed that the study was in accordance with the ethical guidelines of the Helsinki Declaration.

Classification of extracolonic lesions

Extracolonic lesions were divided into three categories, according to previous reports[7,8]. Highly significant lesions include those requiring immediate surgical therapy, medical intervention, and/or further investigation. Examples of highly significant extracolonic lesions include a solid organ mass, adrenal mass greater than 3 cm, aortic aneurysm greater than 3 cm, lymphadenopathy greater than 1 cm, cardiomegaly, pericardial effusion, fistula, abscess and small-bowel infarction.

Lesions of intermediate significance include conditions that do not require immediate therapy but would likely require further investigation, recognition, or therapy at a later time. Examples of such extracolonic lesions include calculi, intermediate cysts, pulmonary fibrosis, inguinal hernia, uterine myoma, endometriosis, pelvic fluid collection, liver cirrhosis, liver hemangioma and bile duct dilatation.

Lesions of low significance include benign conditions that do not require further medical therapy or additional work-up. Examples of such extracolonic lesions include calcifications, granulomas, diverticulosis, simple organ cysts, hernias, pleural thickening, benign prostatic hypertrophy, accessory spleen, benign bony lesion, fatty liver, and renal infarction.

Statistical analyses

Differences between the groups were analyzed using the chi-square test and Student's *t*-test. The age was expressed as mean ± SD (standard deviation) values. Cost effectiveness was calculated by cost needed for detecting one highly significant lesion (cost of CT colonography × total number of CT colonography/number of subjects with highly significant extracolonic lesions). Regression analysis was performed to assess the related factors in detecting extracolonic lesions according to their significance. A probability value of $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the subjects

A total of 920 consecutive subjects (men/women = 535/385) were analyzed. Their mean age (± SD) was 57.3 ± 12.8 (range, 34-87). Of these, 692 extracolonic findings were found in 532 (57.8%) subjects (Table 2). Of the 692 extracolonic findings, 60 (8.7%) were highly significant, 250 (36.1%) were of intermediate significance, and 382 (55.2%) were of low significance (Table 3). Data regarding the examination, age, and sex distribution of each group are summarized in Table 4.

Of 920 subjects, 764 and 156 subjects were examined by CT colonography with and without the aid of contrast enhancement, respectively. Extracolonic lesions were found in 459 of the 764 subjects (60.1%)

Table 1 Characteristics of the study population

Hospital	Number of the subjects	Type of CT colonogrphy	kVp	mAs	Pitch	Slice thickness/reconstruction interval for extracolonic finding (mm)
A	278	Sensation 64; Siemens, Erlangen, Germany	120	70	1.5	3/3
B	157	LightSpeed Ultra 8 or 16; GE Medical systems, Milwaukee, WI	120	70	1.35	1.25/1.25
C	152	Sensation 16; Siemens, Erlangen, Germany	120	30	1	5/5
D	135	Brilliance 40-channel MDCT, Phillips Medical System, Netherlands	120	160	1.176	0.5/0.9
E	92	LightSpeed 16; GE healthcare, Milwaukee, Wis	120	200	1.375	1.25/3.75
F	65	MX 8000 IDT 16, Phillips Eindhoven, Netherlands	120	200	1	2/1
G	41	Sensation 16; Siemens, Erlangen, Germany	120	30	1	5/5

Table 2 Results of 920 computed tomographic colonoscopy examinations *n* (%)

Number of extracolonic findings	Number of subjects
0	388 (42.2)
1	403 (43.8)
2	105 (11.4)
3	19 (2.1)
4	5 (0.5)

examined with contrast enhancement, but in only 73 of the 156 subjects (46.8%) examined without contrast enhancement ($P = 0.005$).

Factors related to the clinical significance of extracolonic findings

The mean age was lower in cases without extracolonic findings (Table 4). With regard to indications for CT colonography, gastrointestinal symptoms were more common in those in whom significant lesions were detected (Table 4). Regression analysis revealed that, older age ($P < 0.001$), being female ($P = 0.001$), presence of symptoms ($P < 0.001$), and the use of contrast during CT colonography ($P = 0.003$) were associated with detection of the more significant extracolonic lesions.

Additional evaluation and management of extracolonic findings

Table 5 lists the additional tests performed in each group. It can be seen that, 81.7% of highly significant subjects received further treatment, while such treatment was received in only 20.8% and 2.9% of subjects of intermediate and low significance, respectively.

Cost of finding a highly significant extracolonic lesion

Since each CT colonography procedure costs US $ 190 (180000 won) in Korea, US $ 2905 (2760000 won) was needed to detect each highly significant lesion in our study (i.e. cost of CT colonography × total CT colonography cases/number of subjects with highly significant extracolonic lesions). The following factors were found to be associated with poor cost-effectiveness: patient age below 60 years, lack of symptoms and use of CT colonography without contrast enhancement (Figure 1).

DISCUSSION

In this study, extracolonic lesions were found in 532 out of 920 subjects (57.8%), which is consistent with previous studies reporting incidences of between 33% and 85%[2-4,6,8,9]. A substantial proportion of these lesions were insignificant, which led to further unnecessary workup and, hence, additional cost. Highly significant extracolonic lesions were detected in the present study

Table 3 Proportion of extracolonic lesions according to the clinical significance

Extracolonic findings	Number
Highly significant (n = 60)	
Solid organ mass including malignancy	42[1]
Cardiomegaly/pericardial effusion	5
Lymphadenopathy greater than 1 cm	3
Peritoneal carcinomatosis	3
Abscess	3
Aortic lesion	2
Small bowel obstruction	2
Intermediately significant (n = 250)	
Benign solid organ lesion	141[2]
Renal stone/hydronephrosis	28
Gall bladder stone/polyp/cholecystitis	22
Liver cirrhosis	13
Bile duct stone/dilatation/hemobilia	9
Small bowel inflammation	8
Vascular lesion (aortic stenosis, varix, *etc*)	6
Bronchiectasis/emphysema	5
Hepatosplenomegaly	5
Pleural effusion	3
Inguinal hernia	3
Ascites of unknown cause	3
Chronic pancreatitis	2
Mesenteric fat necrosis	1
Spinal stenosis with destruction	1
Lowly significant (n = 382)	
Renal cyst	143
Hepatic cyst	114
Fatty liver	39
Vascular calcification/atherosclerosis	19
Chronic pulmonary disease/pleural thickening	16
Accessory spleen/splenic infarction	15
Hepatic calcification	10
Benign osteolytic lesion	8
Hiatal hernia	6
Benign prostatic hypertrophy	5
Colonic diveticulosis	4
Tiny pancreas cyst	1
Mesenteric calcification	1
Gallbladder sludge	1

[1]Liver 9, lung 9, stomach 7, pancreas 3, kidney 3, bladder 3, adrenal gland 2, small bowel 2, bone 1, bile duct 1, psoas muscle 1 and ovary 1. [2]Liver 46, kidney 30, uterus 19, ovary 13, lung 9, adrenal gland 8, lymph node 7, muscle 4, pancreas 3, spleen 1 and testis 1.

Table 4 Baseline characteristics according to the clinical significance of extracolonic lesions *n* (%)

	Highly significant lesion (*n* = 60)	Intermediately significant lesion (*n* = 250)	Lowly significant lesion (*n* = 382)	No extracolonic lesion (*n* = 388)	*P*-value
Age (mean ± SD)	58.3 ± 16.4	57.9 ± 13.8	59.0 ± 11.9	54.4 ± 13.1	< 0.001
Male:Female	36:24	116:134	237:145	233:155	0.001
Indication					< 0.001
Screening	16 (26.7)	76 (30.4)	155 (40.6)	160 (41.2)	
Family history	1 (1.7)	4 (1.6)	4 (1.0)	13 (3.4)	
Past history	10 (16.7)	38 (15.2)	55 (14.4)	93 (24.0)	
GI bleeding	5 (8.3)	19 (7.6)	11 (2.9)	17 (4.4)	
IDA	1 (1.7)	7 (2.8)	8 (2.1)	0 (0.0)	
Bowel habit change	6 (10.0)	27 (10.8)	44 (11.5)	38 (9.8)	
Abdominal pain	17 (28.2)	58 (23.2)	78 (20.4)	56 (14.4)	
Others	4 (6.7)	21 (8.4)	27 (7.1)	11 (2.8)	
CT with enhancement[1]	53 (88.3)	225 (90.0)	320 (83.7)	305 (78.6)	0.001
Hospital					< 0.001
A (*n* = 313)	23 (38.4)	61 (24.4)	77 (20.2)	151 (38.9)	
B (*n* = 214)	9 (15.0)	86 (34.4)	86 (22.6)	33 (8.5)	
C (*n* = 171)	5 (8.3)	24 (9.6)	60 (15.7)	81 (20.9)	
D (*n* = 149)	2 (3.3)	31 (12.4)	70 (18.3)	45 (11.6)	
E (*n* = 104)	10 (16.7)	20 (8.0)	44 (11.5)	30 (7.7)	
F (*n* = 73)	5 (8.3)	16 (6.4)	12 (3.1)	40 (10.3)	
G (*n* = 59)	6 (10.0)	12 (4.8)	33 (8.6)	8 (2.1)	

[1]Computed tomography with pre- and post-contrast images enhanced by intravenous contrast. SD: Standard deviation; GI: Gastrointestinal; IDA: Iron deficiency anemia.

Table 5 Further managements according to the clinical significance of extracolonic lesions *n* (%)

	Highly significant lesion (*n* = 60)	Intermediately significant lesion (*n* = 250)	Lowly significant lesion (*n* = 382)
Diagnostic intervention			
US	6 (10.0)	108 (43.2)	31 (8.1)
CT	17 (28.3)	65 (26.0)	21 (5.5)
MRI	4 (6.7)	14 (5.6)	0 (0.0)
Biopsy	8 (13.3)	3 (1.2)	2 (0.5)
Endoscopy	6 (10.0)	6 (2.4)	1 (0.3)
Other tests	18 (30.0)	25 (10.0)	10 (2.6)
Not done	1 (1.7)	29 (11.6)	317 (83.0)
Therapeutic intervention	49 (81.7)	52 (20.8)	11 (2.9)

US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging.

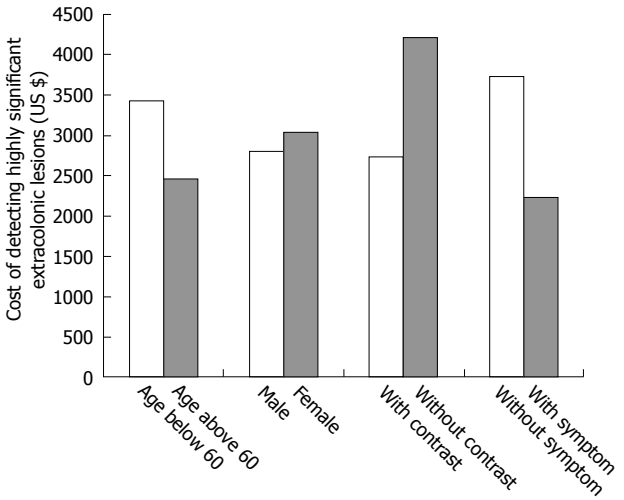

Figure 1 Cost of detecting highly significant extracolonic lesions. Cost-effectiveness was assessed using the following calculation for each group. Poor cost-effectiveness in the detection of highly significant lesions was observed for subjects aged below 60 year-old (US $ 3442), subjects without symptoms (US $ 3737), and CT colonography performed without contrast enhancement (US $ 4221).

in only 60 of 920 subjects (6.5%), which is slightly lower than the incidences found in previous studies. This discrepancy might be due to differences in the study population (ours included only Koreans), the definition of highly significant lesion used and the CT colonography conditions used. In our study, a solid organ mass suspicious of malignancy was detected in 42 of 920 (4.6%) subjects. Considering that substantial numbers of subjects undergoing CT colonography are found to have clinically important extracolonic findings, this would have positive effects on health care by undergoing additional evaluations[10]. The cost of a CT colonography in Korea, i.e. US $190 (180 000 won), is only US $ 53 (50 000 won) more expensive than colonoscopy. Therefore, CT colonography might be more attractive in Korea, since is it less expensive when compared with US[11,12]. Several studies have reported on a prospective cost-benefit analysis of diagnostic CT colonography[10,13]. Some reported low clinical relevant disease in average-risk asymptomatic adults[14], while others revealed higher proportion of colon cancers in subjects with colonic symptoms[13]. We further tried to idebtify the factors associated with the more effective use of CT colonography by analyzing the cost of detecting highly significant extracolonic lesions. As expected, the prevalence of significant extracolonic lesions was higher in older subjects and those with gastrointestinal symptoms. Since our results suggest that significant extracolonic lesions can be anticipated at a higher

frequency in this population than in an asymptomatic younger population, they also contribute toward a better understanding of the selection of subjects who would benefit more effectively from CT colonography.

Apart from age and clinical symptoms, contrast enhancement was found to be advantageous in identifying extracolonic lesions on CT colonography. This demonstrates that some important extracolonic lesions might have been overlooked in non-contrast enhanced cases. The inability of low-radiation dose CT colonography to accurately define lesions in other organs also raises important medico-legal considerations[2]. Based on our findings, age, the presence of gastrointestinal symptoms and the use of contrast enhancement must be taken into account when deciding when to use CT colonography in routine clinical practice.

In the present study, 221 of 250 (88.4%) subjects with extracolonic lesions of intermediate significance were referred for further investigations, of which 52 (20.8%) received treatment, while 65 of 382 (17.0%) subjects with extracolonic lesion of low significance were referred for further investigations, of which only 11 (2.9%) received treatment. Because symptomatic subjects were included in our study, CT colonography was performed as a diagnostic evaluation as well as a screening tool. This would explain why further investigations frequently followed CT colonography. Our results indicate that further investigations pertaining to extracolonic lesions, other than those of high significance, benefit only a few and result in additional and unnecessary cost as a result of unnecessary workups.

The limitation of our study is that there were some differences due to inhomogenous settings. Different participating institutions used such relevant differences in study protocols: Slice thickness varies between 0.5 and 5 mm and mAs varies between 30 and 200. The radiation dose was in the range of 1.7-8.8 mSv, with a median of 3.9 mSv. It was comparatively larger than simple X-ray or plain abdomen with approximately 0.1 mSv. For example, the hospitals D, E and F used almost standard dose contrast, while slice thickness were less than 1 mm for extracolonic lesions at the hospitals B, D and F. However, when considering that the proportions of normal extracolonic findings were highest in hospital F (54.5%) > A (48.2%) > C (47.3%) > D (30.2%) > E (28.8%) > B (15.4%) > G (13.6%), slice thickness and standard dose are not predictive factors of the presence of extracolonic lesions. Another limitation concerns the number of false positive and false negative results of the exam. Since study populations have not been followed up periodically, correct false positivity and negativity could not be evaluated. However, subjects diagnosed as having significant extracolonic lesions received full evaluation and treatment for their lesions. Accordingly, false positivity of significant extracolonic lesions was nearly zero.

In conclusion, most of the extracolonic lesions detected by CT colonography were of low significance, and resulted in additional costly investigations. However, CT colonography may demonstrate asymptomatic malignant disease requiring immediate treatment in older subjects and among those with symptoms, particularly when performed with contrast enhancement. Based on these results, CT colonography should be performed with contrast enhancement in symptomatic older subjects.

COMMENTS

Background

Currently, computed tomographic (CT) colonography is widely used in the clinical field to visualize colon and extracolonic lesions. Extracolonic lesions occur in 15%-85% of cases, with some being important lesions, such as extracolonic cancer or aortic aneurysm. The utilization of CT colonography will increase in clinical field, and research for availability, detection rate and cost-effectiveness of CT colonography is necessary.

Research frontiers

Early detection of extracolonic lesions is an aim of CT colonography. In particular, the detection of significant lesions is very important. However, the incidence rates of significant extracolonic lesions vary from country to country, and most reports relate to the Western population. This is the first study focusing on the Asian population, where the incidence rate of colorectal disease is lower than in the Western population.

Innovations and breakthroughs

In recent reports, cost-effectiveness of CT colonography was calculated in US dollars, because most studies were carried out in the USA. However, the cost of CT colonography varies according to different countries. This study evaluated its cost-effectiveness taking into consideration the specific medical system of the country. In addition, optimal methods to detect significant extracolonic lesions were evaluated. This study showed that the selective use of CT colonoscopy (for symptomatic elderly and with contrast enhancement) shows a good cost-effectiveness.

Applications

Use of CT colonography is currently rising due to its various functions. However, cost of CT colongraphy is comparatively high, and clinical availability is being evaluated. This study is helpful to clinicians to determine the best way to use the CT colonography for detecting highly significant extracolonic lesions.

Terminology

Multi-section helical CT colonography was first introduced in 1998. There have many improvements such as faster scanning, improved temporal resolution, and reduced movement artifacts. Various approaches were tried to increase the effectiveness of CT colonography, and contrast enhancement is recommended as a good strategy if it is applied to an ideal case.

Peer review

The authors examined the cost-effectiveness of CT colonography for various Korean patients, and proposed how to optimize the use of CT colonography. Considering the rising application of CT colonography to the medical field, this study will provide a good basis to guide its use.

REFERENCES

1 **Burling D**, Taylor SA, Halligan S. Virtual colonoscopy: current status and future directions. *Gastrointest Endosc Clin N Am* 2005; **15**: 773-795

2 **Edwards JT**, Wood CJ, Mendelson RM, Forbes GM. Extracolonic findings at virtual colonoscopy: implications for screening programs. *Am J Gastroenterol* 2001; **96**: 3009-3012

3 **Hellström M**, Svensson MH, Lasson A. Extracolonic and incidental findings on CT colonography (virtual colonoscopy). *AJR Am J Roentgenol* 2004; **182**: 631-638

4 **Hara AK**, Johnson CD, MacCa-rty RL, Welch TJ. Incidental extracolonic findings at CT colonography. *Radiology* 2000; **215**: 353-357

5 **Rockey DC**. Colon imaging: computed tomographic

colonography. *Clin Gastroenterol Hepatol* 2005; **3**: S37-S41
6 **Ginnerup Pedersen B**, Rosenkilde M, Christiansen TE, Laurberg S. Extracolonic findings at computed tomography colonography are a challenge. *Gut* 2003; **52**: 1744-1747
7 **Sosna J**, Kruskal JB, Bar-Ziv J, Copel L, Sella T. Extracolonic findings at CT colonography. *Abdom Imaging* 2005; **30**: 709-713
8 **Gluecker TM**, Johnson CD, Wilson LA, Maccarty RL, Welch TJ, Vanness DJ, Ahlquist DA. Extracolonic findings at CT colonography: evaluation of prevalence and cost in a screening population. *Gastroenterology* 2003; **124**: 911-916
9 **Rajapaksa RC**, Macari M, Bini EJ. Prevalence and impact of extracolonic findings in patients undergoing CT colonography. *J Clin Gastroenterol* 2004; **38**: 767-771
10 **Yee J**, Kumar NN, Godara S, Casamina JA, Hom R, Galdino G, Dell P, Liu D. Extracolonic abnormalities discovered incidentally at CT colonography in a male population. *Radiology* 2005; **236**: 519-526
11 **Vijan S**, Hwang I, Inadomi J, Wong RK, Choi JR, Napierkowski J, Koff JM, Pickhardt PJ. The cost-effectiveness of CT colonography in screening for colorectal neoplasia. *Am J Gastroenterol* 2007; **102**: 380-390
12 **Ng CS**, Freeman AH. Incidental lesions found on CT colonography: their nature and frequency. *Br J Radiol* 2005; **78**: 20-21
13 **Khan KY**, Xiong T, McCafferty I, Riley P, Ismail T, Lilford RJ, Morton DG. Frequency and impact of extracolonic findings detected at computed tomographic colonography in a symptomatic population. *Br J Surg* 2007; **94**: 355-361
14 **Pickhardt PJ**, Taylor AJ. Extracolonic findings identified in asymptomatic adults at screening CT colonography. *AJR Am J Roentgenol* 2006; **186**: 718-728

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Hirschsprung's disease: Is there a relationship between mast cells and nerve fibers?

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Abstract

AIM: To define the topography of mast cells and their numbers in cases of Hirschsprung's disease (HD) and non-HD, assess neural hypertrophy using imaging software and to study the relationship between mast cells and nerve fibers.

METHODS: HE stained sections of 32 cases of chronic constipation in the age group of 0-14 years were reviewed for ganglion cells. AChE staining was performed on frozen sections of colonic and rectal biopsies. Based on their findings cases were divided into HD and non-HD and mast cells stained by toluidine blue were evaluated. Image analysis by computerized software was applied to S-100 stained sections for assessment of neural hypertrophy.

RESULTS: Difference between number of mast cells in HD group (mean = 36.44) and in non-HD group (mean = 14.79) was statistically significant. Image analysis morphometry on S-100 stained sections served as a useful adjunct. The difference between number, size, and perimeter of the nerve fibers between HD and non-HD group was statistically significant.

CONCLUSION: Mast cells are significantly increased in HD and their base line values are much higher in Indian children than that reported in Western literature. Their role in HD needs further research. Morphometry of S-100 stained nerve fibers is a useful adjunct to conventional methods for diagnosis of HD.

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Key words: Hirschsprung's disease; Mast cells; S-100; Acetylcholinesterase

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INTRODUCTION

Constipation is a common presenting complaint in children attending the pediatric outpatient department[1]. Some 13%-15% cases of constipation are due to aganglionosis of bowel segment or Hirschsprung's disease (HD). HD is a serious disorder and can be fatal if left untreated. The disease is however, surgically correctable, underscoring the need to identify these cases.

HD has an incidence of 1 in 5000 neonates in Western literature and a male to female predominance of 4:1. Although there are no statistical figures available from India, a large number of studies in Indian literature suggest that the disorder is not uncommon in our country[2-4].

Of late there has been a lot of interest in the role of mast cells (MC) in the pathogenesis of Hirschsprung's disease. Kobayashi *et al*[5] have found an increased number of mast cells in aganglionic bowel segments and in those with intestinal neuronal dysplasia. The exact role of mast cells in HD is not known. Therefore we undertook this study to evaluate the relationship between mast cells and nerve fibers in HD cases to shed some light on this issue. We have used image analysis morphometry to analyze nerve fibers stained with S-100 and correlated the findings with mast cell numbers.

There are only a couple of studies on mast cells in

HD in the literature[5-7]. There is however no study from India on mast cells in children with HD or constipation. As children in a developing country like India are more likely to be exposed to inflammatory stimuli like bacterial and viral antigens and pollutants/allergens as compared to their Western counterparts, an increase in mast cells was expected in the intestines. The cells were assessed in children with constipation, with an emphasis on HD, to ascertain their distribution in the colonic and rectal biopsies.

MATERIALS AND METHODS

The study was conducted in the departments of Pathology and Surgery, University College of Medical Sciences and GTB Hospital, New Delhi.

Selection criteria

Thirty two cases of chronic constipation (defined as decreased frequency of bowel movements "fewer than three each week" or a difficulty in defecation perceived by the parents as a problem that requires medication or manual intervention[1]) in the age group of 0-14 years were enrolled for the study. Cases of constipation due to local causes like anal fissure, anal stenosis and anterior perineal anus were excluded from the study.

The criterion used for diagnosis of HD in all of the HD cases was absence of ganglion cells upon evaluation of Haematoxylin & Eosin (HE) stained sections of colonic and rectal biopsies. Acetylcholinesterase (AChE) stain for the nerve fiber pattern was carried out in 12 cases. Based on the findings of HE and AChE they were divided into two groups: Group Ⅰ - HD and Group Ⅱ -non-HD. Group Ⅰ -HD included 11 rectal biopsies and 7 resection specimens. Group Ⅱ-non-HD included 14 rectal biopsies. Rectal biopsies obtained were rectal punch biopsies. Mucosa and submucosa was available for review in all the cases. Muscularis propria was available in resection specimens only.

Toluidine blue stain was performed on paraffin sections of all the cases to evaluate mast cells. Immunohistochemical stain for S-100 was also performed on 12 HD cases & 8 non-HD cases.

Acetylcholinesterase stain

Ten micrometer thick sections cut on the cryostat were fixed by dipping in buffered formalin for 1-2 min, preserved by wrapping them in aluminium foil and stored at -20℃ in a deep freezer till the time of staining. Stock solutions of various ingredients of incubation medium were kept at 4℃ and the incubation medium was prepared just before staining. AChE stain was performed using modified Karnovsky and Roots method as described previously[8]. Incubation medium was prepared by mixing 0.1 mol/L sodium hydrogen maleate buffer 6.5 mL (pH 6.0), 0.1 mol/L sodium citrate 0.5 mL, 30 mmol/L copper sulphate 1.0 mL, 5 mmol/L potassium ferricyanide 1.0 mL, water 1.0 mL and acetylthiocholine iodide 5 mg. The sections were incubated at 37℃ for 20 min in the incubation medium. Thereafter, the incubation medium was drained off and re-incubation with rubeanic acid solution was performed for 10 min. Rubeanic acid solution was prepared by mixing absolute alcohol 10 mL, sodium acetate 6.55 g, rubeanic acid 10 mg and distilled water 40 mL. Thereafter, the sections were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX.

Toluidine blue

Toluidine blue staining on paraffin sections was performed using a simple toluidine blue method that required incubation of sections in 1% aqueous solution of toluidine blue and mounting in a water based medium. Mast cells were counted in five successive high power fields (× 400) in the submucosa.

S-100 Stain

Immunohistochemical stain for S-100 (Dako Corporation) was performed on HD (12 cases) and non-HD (8 cases) by the standard immunoperoxidase technique. The nerve fibers that stained positive for S-100 were counted in five high power fields (× 400) in the submucosa.

Image analysis

Images of relevant areas in S-100 stained slides were captured using a digital camera. They were subsequently analyzed using the Scion image analysis software (www.scioncorp.com). The size and perimeter of the thickest nerve fiber in the submucosa was measured. The number, size and perimeter of nerve fibers were correlated with mast cell number in both the groups.

Statistical analysis

Statistical analysis was performed by the chi-square and student *t*-test and correlation assessed by Pearson's correlation coefficient.

RESULTS

A total of 32 cases of chronic constipation in children below 14 years were studied. Based on the findings of HE and AChE stained sections of colonic and rectal biopsies, they were divided into two groups: Group Ⅰ - HD and Group Ⅱ -non-HD. Group Ⅰ -HD included 18 cases and Group Ⅱ -non-HD included 14 cases.

The age of the patients ranged from 1 mo to 84 mo in the HD group (mean ± 2 SD = 26.78 ± 36.72 mo). Maximum numbers of cases were found in the age group 36-47 mo in this group. In the non-HD group, age ranged from 6 d to 96 mo (mean ± 2 SD = 26.64 ± 49.60 mo), maximum cases in this group being in the age group of 0-11 mo. Male to female ratio was 17:1 in the HD group and 4:3 in the non-HD group.

There was statistically no significant difference in the age distribution between the two groups ($P = 0.8$). The difference in sex distribution between the two groups was statistically significant, the patient population in the HD group having a distinct male bias ($P = 0.01$).

Mast cells

The data obtained after evaluation for mast cells are

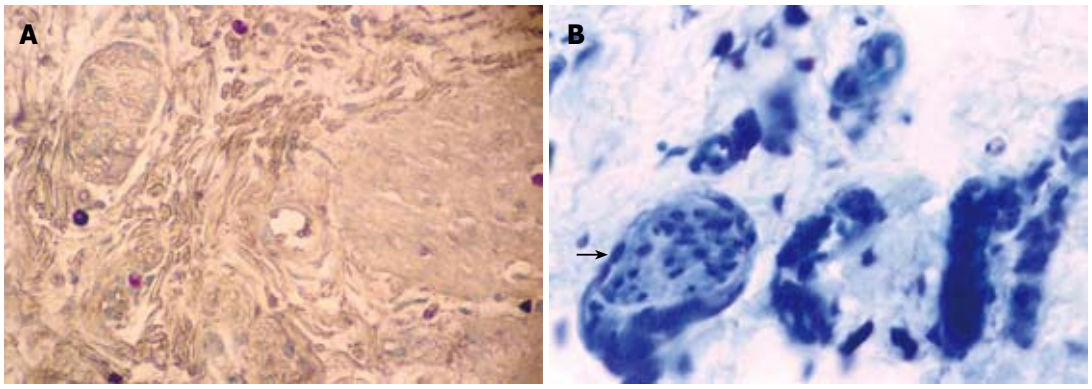

Figure 1 Perineural and intraneural mast cells (× 200) are increased in HD patients (A) compared to non-HD patients (B). (Toluidine blue, × 200).

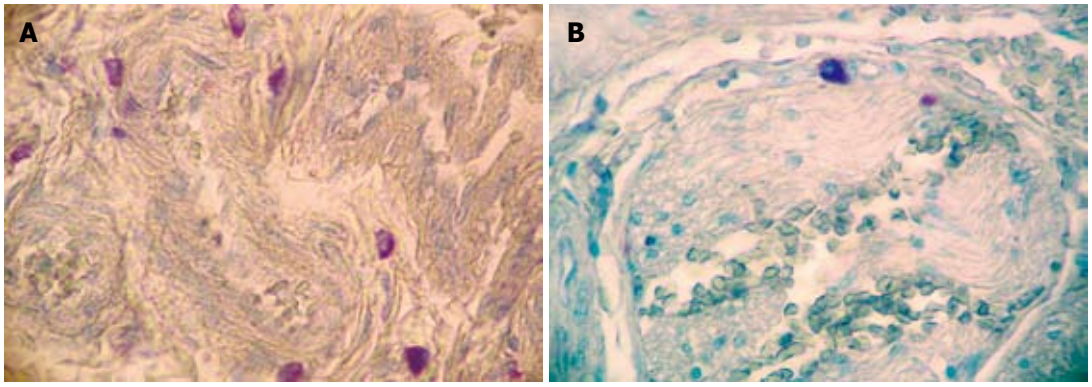

Figure 2 Increased number of mast cells in perivascular distribution (× 400) in HD case (A) as compared to non-HD case (B) showing an occasional mast cell (× 400) only. (Toluidine blue).

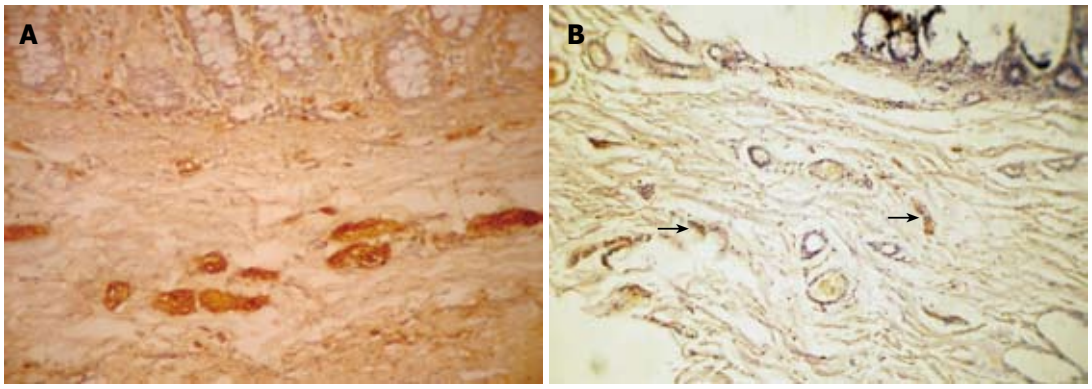

Figure 3 Hypertrophic nerve fibers in submucosa (× 100) in HD case (A) as compared to small and thin nerve fibers (black arrows, × 100) in non-HD case (B). (S-100).

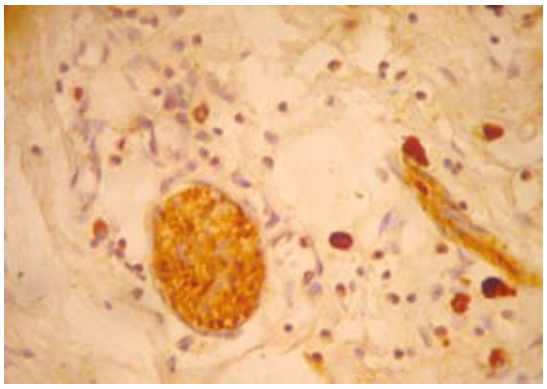

Figure 4 Hypertrophic nerve fiber in submucosa surrounded by mast cells. (S-100, × 400).

summarized in Table 1. There was a statistically significant difference in the number of MC between HD (mean ± 2 SD = 36.44 ± 36.02) and non-HD (mean ± 2 SD = 14.79 ± 19.8) (P = 0.0001). The mast cells were distributed transmurally (An observation in resection specimens of HD cases. Statistical analysis could not be done due to small sample size) and were notably present around the nerve fibers (Figure 1) and perivascularly (Figure 2). The number of mast cells in HD as well as in non-HD cases was significantly higher than previous studies (Table 2).

S-100

S-100 immunohistochemistry was performed on 20 cases consisting of 12 HD and 8 non-HD cases (Table 3). The number of nerve fibers in the submucosa (Figure 3) of HD (mean ± 2 SD = 11.17 ± 6.18) cases was higher than in non-HD (mean ± 2 SD = 5.13 ± 7.66) cases. The difference between the two groups was statistically significant (P = 0.0001). S-100 also stained the mast cells (Figure 4).

Image analysis

Size of the nerve fibers in HD group was mean ± 2 SD

Table 1 Mast cells in Hirschsprung's disease (HD) and non-HD cases

	HD cases				Non-HD cases		
S. No.	Age (mo)	Sex	No. of mast cells	S. No.	Age (mo)	Sex	No. of mast cells
1	36	M	35	1	30	F	1
2	36	M	40	2	24	M	15
3	12	F	35	3	36	M	25
4	24	M	35	4	36	F	5
5	3	M	10	5	4	M	10
6	36	M	30	6	48	M	3
7	5	M	50	7	1	M	1
8	5	M	20	8	1	M	25
9	36	F	70	9	96	M	15
10	24	F	20	10	1	F	32
11	84	M	45	11	2	M	15
12	36	M	45	12	30	F	25
13	84	M	80	13	36	M	15
14	18	F	30	14	48	M	20
15	36	M	45				
16	48	M	26				
17	48	M	13				
18	12	F	27				

HD (mean ± 2 SD = 36.44 ± 36.02) and non-HD (mean ± 2 SD = 14.79 ± 19.8) [(HD *vs* non-HD) $P = 0.0001$].

= 57.18 ± 24.86 μm and in non- HD group it was mean ± 2 SD = 31.03 ± 39.08 μm. The difference between the two was statistically significant ($P = 0.0001$). The perimeter of nerve fiber in HD group was mean ± 2 SD = 201.89 ± 88.78 μm and in non-HD group was mean ± 2 SD = 113.26 ± 141.18 μm. This difference between the two groups was also statistically significant ($P = 0.0001$) (Table 2).

Mast cells and nerve fibers

We correlated each of the parameters obtained by image analysis morphometry of the nerve fibers with MC number. In the HD group it was observed that maximum correlation was seen with number of nerve fibers (correlation coefficient = 0.467). No correlation was seen with size of nerve fibers (correlation coefficient = -0.131) and some correlation was observed with the perimeter of nerve fibers (correlation coefficient = 0.274). In the non-HD group the respective values were 0.406, 0.304 and 0.157.

DISCUSSION

Recently there has been a lot of interest in the role of mast cells in HD. Kobayashi *et al*[5] described an increased number of mast cells in the aganglionic segment of the colon in patients with HD. The number of these cells in transitional segments was significantly less compared with ganglionic segments in HD patients and controls. Similar findings were reported by Demirbilek *et al*[6].

In the present study, mast cells were evaluated in rectal biopsies as well as in resected specimens from cases of HD and non-HD. Although the mast cells can be roughly estimated after HE staining, an exact count is obtained by special histochemical methods. Other authors[5] have used immunohistochemical detection of anti-MC antibody to demonstrate MC. In our study, these were studied by a simple and effective toluidine blue staining which requires a time as short as one minute incubation. A significant increase was noticed in the number of mast cells in HD as compared with the non-HD cases. The HD cases showed a transmural distribution of these cells as described in the previous two studies by Kobayashi *et al*[5] and Demirbilek *et al*[6]. However, in a recent study Hermanowicz *et al*[7] found them to be increased in the mucosa and lamina propria but the increase in mast cells in the submucosa, muscularis propria and serosa was not statistically significantly changed.

Table 2 Comparison of mast cells in submucosa of HD and non-HD cases with previous studies

Mast cells per 5 hpf in submucosa		Kobayashi *et al*[5]	Demirbilek *et al*[6]	Present study
HD cases	Aganglionic segment	23.9 ± 6.6	18.2 ± 3.3	36.36 ± 39.58
	Ganglionic segment	8.5 ± 3.9	1.7 ± 0.4	38 ± 16.66
Non-HD cases		7.2 ± 3.4	5.4 ± 1.2	14.8 ± 5.3

Aganglionic segment (Kobayashi *et al vs* Present study, $P = 0.0001$; Demirbilek *et al vs* Present study, $P = 0.0001$); Ganglionic segment (Kobayashi *et al vs* Present study, $P = 0.0001$; Demirbilek *et al vs* Present study, $P = 0.0001$); Non HD cases (Kobayashi *et al vs* Present study, $P = 0.0001$; Demirbilek *et al vs* Present study, $P = 0.0001$).

The mast cells were characteristically distributed around the nerves and blood vessels in addition to being randomly scattered. In our study the baseline number of the mast cells (i.e. number of mast cells in non-HD cases) was much higher than previous studies[5,6] (Table 2) and few mast cells were also seen in the ganglionic segment. This may be due to the response of the mast cells to infectious agents or allergens rather than their association with aganglionosis. An increased number of mast cells is also reported in various other gastrointestinal disorders such as acute appendicitis, ulcerative colitis, celiac disease and gluten enteropathy[9,10]. They are seen to be in apposition to the nerves[11] and are known to secrete substances like nerve growth factor[12].

We also correlated each of the parameters obtained by image analysis morphometry of the nerve fibers with MC number. It was observed that maximum correlation was seen with number of nerve fibers (correlation coefficient = 0.467). No correlation was seen with size of nerve fibers and some correlation was observed with the perimeter of nerve fibers (correlation coefficient = 0.274). This correlation has not been shown in any previous study. This suggests that mast cells via their mediators may cause increased number of nerve fibers and affect size of nerve fibers to some extent. The interactive role of mast cells, their nerve growth factor secretion and neural hypertrophy as suggested by morphology is not explained by the studies to date.

Table 3 Number, size and perimeter of nerve fibers in HD and non-HD cases

	HD cases					Non-HD cases				
S. No.	Age (mo)	Sex	No. of nerve fibers	Size of nerve fibers (μm)	Perimeter of nerve fibers (μm)	Age (mo)	Sex	No. of nerve fibers	Size of nerve fibers (μm)	Perimeter of nerve fibers (μm)
1	42	M	13	40.37	230.48	36	M	3	42.5	215.7
2	12	F	8	68.0	195.9	1	M	2	11.2	37.0
3	36	M	11	65.4	172.8	5	M	4	15.3	47.3
4	84	M	7	47.3	135.4	1	M	4	15.0	51.8
5	48	M	16	63.5	288.75	1	F	3	40.6	135.56
6	36	M	10	77.38	237.79	30	F	3	25.0	126.67
7	48	M	12	57.5	256.11	4	M	9	28.8	83.2
8	36	M	14	40.7	149.3	13	M	13	69.9	208.09
9	12	M	6	55.5	190.03					
10	24	M	11	47.6	184.08					
11	1	M	15	49.6	178.18					
12	60	M	11	73.4	203.9					

Number of nerve fibers (HD *vs* non-HD), $P = 0.0001$; Size of nerve fibers (HD *vs* non-HD), $P = 0.0001$; Perimeter of nerve fibers (HD *vs* non-HD), $P = 0.0001$.

The exact role of these cells in the pathogenesis of HD needs further research with focus on the enteric nervous system, its development and the role of MC in cases where ganglion cells are absent.

The neural hypertrophy in the colonic submucosa is associated with aganglionosis and is a surrogate marker for the disease. The thickness of submucosal nerve fibers has been measured by various authors in previous studies[13,14]. However, they have used less objective methods for measurement such as image graticules. In this study, we analyzed the status of the nerve fibers after S-100 immuno-staining and examination using an objective technique provided by image analysis software. The number, size and perimeter of the nerve fibers in the colonic submucosa of HD and non-HD cases were measured and results compared between HD and non-HD cases. The difference between the two groups was statistically significant for all the three parameters. Thus image analysis can be a useful adjunct to the available tools for the diagnosis of HD.

In conclusion, mast cells appear to have a significant role in the pathogenesis of Hirschsprung's disease. Their increased baseline number in Indian children may be a response to additional factors like allergens or antigenic components of infectious agents. The role of anti-mast cell reagents like sodium cromoglycate needs to be explored with regard to constipation in Indian children. The fact that Indian children with HD present at a later age is also significant and emphasizes the need for a different approach to disease in various geographical regions.

COMMENTS

Background

Hirschsprung's disease is an important cause of constipation in children. It is a serious disorder and can be fatal if left untreated. Recently, there has been a lot of interest in the role of mast cells in the pathogenesis of Hirschsprung's disease. A few studies have found increased number of mast cells in aganglionic bowel segments of Hirschsprung's disease cases. However the exact role of mast cells in Hirschsprung's disease is not known.

Research frontiers

Mast cells are normally present in small numbers in various organs of the body. They have been reported to be increased in a number of conditions other than Hirschsprung's disease. Regarding the role of mast cells in Hirschsprung's disease, the research hotspot is to elucidate the exact role played by them in the pathogenesis of Hirschsprung's disease and the relationship between mast cells and the neural hypertrophy observed in Hirschsprung's disease.

Innovations and breakthroughs

Previous studies on the role of mast cells in the pathogenesis of Hirschsprung's disease have described an increased number of mast cells in the aganglionic segment in patients with Hirschsprung's disease. Apart from one article, other studies have found mast cells to be increased transmurally. In the present study a significant increase was also noticed in mast cells in Hirschsprung's disease. However their number was significantly higher than previous studies. This may be due to the response of the mast cells to infectious agents or allergens rather than their association with aganglionosis. The authors also performed image analysis morphometry and correlated each of the parameters obtained with mast cell number. It was observed that maximum correlation was seen with number of nerve fibers. No correlation was seen with size of nerve fibers and some correlation was observed with the perimeter of nerve fibers. This correlation has not been shown in any previous study. This suggests that mast cells via their mediators may cause increased number of nerve fibers and affect size of nerve fibers to some extent.

Applications

The study suggests mast cells appear to have a significant role in the pathogenesis of Hirschsprung's disease. Whether this fact can be exploited to open novel therapeutic options in the management of this disease is something which needs to be looked at in the future.

Terminology

Hirschsprung's disease is a congenital condition characterized by dilatation and hypertrophy of colon due to absence (aganglionosis) or marked reduction (hypoganglionosis) of ganglion cells of the myenteric plexus of the rectum and a varying but continuous length of colon above the rectum. Mast cells are connective tissue cells with cytoplasmic coarse metachromatic granules and their normal function is unknown.

Peer review

This article only contributes minimal new information to the literature (morphometry or S-100 stained nerve fibers), but it reinforces the observation that mast cells are increased in patients with HD and reports this finding in Indian children for the first time.

REFERENCES

1 **Ghosh A**, Griffiths DM. Rectal biopsy in the investigation of constipation. *Arch Dis Child* 1998; **79**: 266-268
2 **Agarwala S**, Bhatnagar V, Mitra DK. Long-term follow-up of Hirschsprung's disease: review of early and late complications. *Indian Pediatr* 1996; **33**: 382-386
3 **Bajpai M**, Lall A. Surgical aspects of chronic constipation in children. *Indian J Pediatr* 1999; **66**: S89-S93

4 **Vijaykumar**, Chattopadhyay A, Patra R, Murulaiah M. Soave procedure for infants with Hirschsprung's disease. *Indian J Pediatr* 2002; **69**: 571-572
5 **Kobayashi H**, Yamataka A, Fujimoto T, Lane GJ, Miyano T. Mast cells and gut nerve development: implications for Hirschsprung's disease and intestinal neuronal dysplasia. *J Pediatr Surg* 1999; **34**: 543-548
6 **Demirbilek S**, Ozardali HI, Aydm G. Mast-cells distribution and colonic mucin composition in Hirschsprung's disease and intestinal neuronal dysplasia. *Pediatr Surg Int* 2001; **17**: 136-139
7 **Hermanowicz A**, Debek W, Dzienis-Koronkiewicz E, Chyczewski L. Topography and morphometry of intestinal mast cells in children with Hirschsprung's disease. *Folia Histochem Cytobiol* 2008; **46**: 65-68
8 **Goto S**, Ikeda K, Toyohara T. An improved staining technique for acetylcholinesterase activity using rubeanic acid in the diagnosis of Hirschsprung's disease. *Jpn J Surg* 1984; **14**: 135-138
9 **Stead RH**, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. *Gastroenterology* 1989; **97**: 575-585
10 **Krishnaswamy G**, Kelley J, Johnson D, Youngberg G, Stone W, Huang SK, Bieber J, Chi DS. The human mast cell: functions in physiology and disease. *Front Biosci* 2001; **6**: D1109-D1127
11 **Stenton GR**, Vliagoftis H, Befus AD. Role of intestinal mast cells in modulating gastrointestinal pathophysiology. *Ann Allergy Asthma Immunol* 1998; **81**: 1-11; quiz 12-15
12 **Leon A**, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci USA* 1994; **91**: 3739-3743
13 **Petchasuwan C**, Pintong J. Immunohistochemistry for intestinal ganglion cells and nerve fibers: aid in the diagnosis of Hirschsprung's disease. *J Med Assoc Thai* 2000; **83**: 1402-1409
14 **Monforte-Munoz H**, Gonzalez-Gomez I, Rowland JM, Landing BH. Increased submucosal nerve trunk caliber in aganglionosis: a "positive" and objective finding in suction biopsies and segmental resections in Hirschsprung's disease. *Arch Pathol Lab Med* 1998; **122**: 721-725

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Expression of phosphatase regenerating liver 3 is an independent prognostic indicator for gastric cancer

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Abstract

AIM: To investigate the prognostic significance of phosphatase regenerating liver 3 (PRL-3) protein expression in gastric cancer.

METHODS: PRL-3 expression in paraffin-embedded tumor specimens from 293 patients with gastric cancer was studied retrospectively by immunohistochemistry. Monoclonal antibody specifically against PRL-3, 3B6, was obtained with hybridoma technique.

RESULTS: Positive PRL-3 expression was detected in 43.3% (127 of 293) of gastric cancer cases. High expression of PRL-3 was positively correlated with tumor size, depth of invasion, vascular/lymphatic invasion, lymph node metastasis, high TNM stage and tumor recurrence. Patients with positive PRL-3 expression had a significantly lower 5-year survival rate than those with negative expression (28.3% *vs* 51.9%, $P < 0.0001$). Patients who received curative surgery, and with positive PRL-3 expression had a significant shorter overall survival and disease-free disadvantage over patients with negative expression (hazard ratio of 16.7 and 16.6, respectively; $P < 0.0001$ for both). Multivariate analysis revealed that PRL-3 expression was an independent prognostic indicator for overall and disease-free survival of gastric cancer patients, particularly for survival in TNM stage Ⅲ patients.

CONCLUSION: PRL-3 expression is a new independent prognostic indicator to predict the potential of recurrence and survival in patients with gastric cancer at the time of tumor resection.

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Key words: Phosphatase regenerating liver 3; Gastric cancer; Prognosis; Recurrence; Antibody

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies in the world with a high incidence and death rate. In China, it remains the most frequent cancer and the second cancer-related cause of death with a high case fatality[1]. TNM staging system is used worldwide to predict prognosis and direct therapeutic decisions of patients with GC. The 5-year survival rate in patients with stage Ⅰ GC is close to 90% and around 10% for patients with stage Ⅳ GC[2]. However, the prognoses of patients with stage Ⅱ and Ⅲ GC are more heterogeneous and less predictable by staging criteria. Therefore, finding molecular markers that are able to predict the potential of tumor recurrence and prognosis of patients is extremely important for appropriate individualized therapy. Phosphatase regenerating liver 3 (PRL-3) (also known as PTP4A3) belongs to a newly

discovered group of phosphotase regenerating liver family which is implicated in oncogenic and metastatic processes[3,4]. The PRL family represents a protein tyrosine phosphatase superfamily possessing a unique COOH-terminal prenylation motif and a protein tyrosine phosphatase-active site signature sequence CX5R[5,6]. Three PRLs (PRL-1, -2, and -3) are highly homologous with similar amino acid sequence of 76%-87%[7-9]. Recent reports found that PRL-3 was consistently expressed at higher level of metastasis in liver compared to normal colorectal epithelia and primary cancer tissues[10,11]. A growing body of evidence showed that an excess of PRL-3 phosphatase is a key alteration contributing to the acquisition of metastatic properties of the tumor cells. For example, nontumorigenic or low metastatic cell lines transfected with wild type PRL-3 displayed higher cell motility and invasiveness and could induce metastatic tumor formation in mice, while cells expressing catalytically inactive mutant PRL-3 significantly reduced the migratory capability[12,13]. Knockdown of endogenous PRL-3 in cancerous cells using small interfering RNA or phosphatase inhibitors can abrogate cell motility and the ability to form metastasis-like tumors in mice[12-15]. PRL-3 was further demonstrated to be a useful indicator for tumor recurrence and patient outcome in several human cancers including colorectal cancer and breast cancer[16-19]. In gastric cancer, PRL-3 was found to be highly expressed in tumor metastatic lymph nodes and closely associated with the peritoneal metastasis[20-23], but the prognostic impact of PRL-3 expression in gastric cancer still remains to be further investigated.

In this study, we detected the expression of PRL-3 in GC tissue samples by immunohistochemistry using a PRL-3 specific monoclonal antibody 3B6 to investigate PRL-3 protein expression in GC tissues and whether PRL-3 could be applied as a prognostic indicator for GC to predict the potential of tumor recurrence and patient outcome.

MATERIALS AND METHODS

Patients

This retrospective study enrolled patients who underwent clinical surgery for primary gastric cancer at the Department of Surgery, Beijing Cancer Hospital, Peking University School of Oncology between July 1994 and December 2000. Patients with inadequate histologic specimens or missing clinical information were excluded. A total of 293 patients were finally included. There were 194 males and 99 females, with ages ranging from 25 to 82 (mean ± SD, 58 ± 17.1 years). Two hundred (68.3%) patients received curative resection (R0) with radical lymph node dissection; the remaining 93 (31.7%) patients with microscopic or macroscopic tumor residues were given palliative resection (R1/R2). Site distribution of the primary tumor was 153 at antrum, 52 at cardia or fundus, and 88 at corpus. Tumor size ranged from 5 to 120 mm (mean, 43.8 mm).

Histology

Data were collected from clinical case report record and follow-up database. Tumor staging was based on the clinical evaluation and postoperative pathological reports. TNM staging was on the basis of the 1997 fifth edition of AJCC/UICC TNM staging criteria for gastric cancer[24]. The tumors were histologically classified according to the WHO classification criteria.

Follow-up

None of these patients had received radiotherapy or chemotherapy preoperatively. All the patients were followed up at regular intervals of 6 mo after surgery until June 2006 with a minimum of five years. Tumor recurrence was clinically defined as the reappearance of tumor after curative surgery. The overall survival time was calculated from the date of surgery to the date of last visit or death and the disease-free survival time from the date of resection to relapse.

Immunostaining of PRL-3

Tumor tissue specimens from the 293 patients were routinely fixed in 10% formalin and embedded with paraffin. Paraffin embedded tissue samples were cut into 4 μm sections. The sections were put in an oven at 60℃ for 5 h and cooled down overnight before they were deparaffinized in xylene. The sections were then dehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwaving the sections in 1 mmol/L EDTA (PH 8.0) for 15 min. PRL-3 monoclonal antibody 3B6 (a generous gift from Prof. Shou, Beijing Institute for Cancer Research, China)[25] was used as the primary antibody at a dilution of 1:100 overnight at 4℃. The Powervision two-step histostaining reagent PV-6002 (Dako, Glostrop, Denmark) was applied as the secondary antibody. The sections were visualized with diaminobenzidine and counterstained with hematoxylin. Each incubation step was followed by washing with phosphate-buffered saline. For negative control, the primary antibody was omitted from the reaction sequence. Sections of liver metastasis from colon cancer with known strong PRL-3 immunoreactivity were used as positive controls. The number of tumor cells with cytoplasm staining of PRL-3 was counted without knowledge of the clinicopathological data, and > 5% positive tumor cells were defined as positive PRL-3 expression[19].

Statistical analysis

Statistical analyses were performed with SAS 8.1 software. The χ^2 test was used to analyze the association between PRL-3 expression and clinicopathological features of GC. Cumulative survival rates and differences in survival curves were estimated by Kaplan-Meier method with the log-rank test. The effect of PRL-3 on survival was analyzed using the Cox proportional hazard regression model adjusted for clinical and histopathologic features. Two-sided *P* values

Table 1 Association between PRL-3 expression and clinicopathological features

Factors	Patients	PRL-3 expression		*P* value
		Positive (*n* = 127)	Negative (*n* = 266)	
Gender				
Male	194	85	109	NS
Female	99	42	57	
Age (yr)				
< 60	149	66	83	NS
⩾ 60	144	61	83	
Tumor size (cm)				
⩾ 5	118	63	55	0.004
< 5	175	64	111	
Depth of invasion				
T1	22	5	17	0.008[a]
T2	46	15	31	0.009[b]
T3	175	77	98	0.042[c]
T4	50	30	20	
Lymph node metastasis				
N0	82	30	52	< 0.0001[d]
N1	120	42	78	
N2	51	33	18	
N3	40	22	18	
Distant metastasis				
Present	42	21	21	NS
Absent	251	106	145	
Vascular/lymphatic invasion				
Present	162	89	73	< 0.0001
Absent	131	38	93	
Differentiation				
Poor	194	91	103	NS
Well/Moderate	99	36	63	
Surgical curability				
Curative	200	79	121	NS
Not curative	93	48	45	
Recurrence[e]				
Absent	177	63	114	0.002
Present	23	16	7	
TNM stages				
Ⅰ	42	13	29	0.007[f]
Ⅱ	52	17	35	0.008[g]
Ⅲ	99	43	56	
Ⅳ	100	54	46	

NS: Not significant; [a]T1-T2 *vs* T3-T4; [b]T1-T3 *vs* T4; [c]T1 *vs* T2-4; [d]N0-N1 *vs* N2-N3; [e]200 cases received curative surgery; [f]Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ; [g]Ⅰ-Ⅲ *vs* Ⅳ.

of less than 0.05 were considered to be statistically significant.

RESULTS

Patient outcome

Forty-two patients were classified as stage Ⅰ, 52 as stage Ⅱ, 99 as stage Ⅲ and 100 as stage Ⅳ. A total of 194 cases were poorly differentiated, 69 cases were moderately differentiated and the remaining 30 cases were well differentiated.

The follow-up period for survivors ranged from 2 to 120 mo (median, 31 mo). The 5-year overall survival rate was 41.7% in the entire cohort of patients, 92.9% in stage Ⅰ, 72.5% in stage Ⅱ, 32.5% in stage Ⅲ and 12.3% in stage Ⅳ patients. One hundred and five patients remained alive and disease-free, 15 patients were alive with disease. One hundred and seventy patients died of GC, and 3 patients died of other causes. Among the 200 patients who received curative surgery, 23 patients had tumor recurrence with 3 in peritoneum, 3 in lymph node, 7 in liver, 3 in other organs (2 ovarian, 1 lung), 4 in multiple organs and 3 in remnant stomach.

PRL-3 expression in GC and its relation with clinicopathological features

PRL-3 immunostaining was predominantly localized in the cytoplasm of normal or tumor epithelial cells. PRL-3 stained cells in normal epithelia were mainly observed in the neck of gastric glands (Figure 1). Among the 293 GC specimens analyzed, 127 (43.3%) tumors had positive PRL-3 expression. The rate of positive PRL-3 expression was significantly higher in stage Ⅲ and Ⅳ than in stage Ⅰ and Ⅱ (48.7% *vs* 31.9%, $P = 0.007$). High expression of PRL-3 was correlated closely with large tumor size, depth of invasion in gastric wall, lymph node metastasis, vascular/lymphatic invasion and recurrent frequency. No significant correlation was observed between PRL-3 expression and sex, age, distant metastasis, grade of differentiation and surgical curability (Table 1).

Univariate survival analysis of prognostic impact of PRL-3 expression

Kaplan-Meier method with log-rank test revealed that patients with positive PRL-3 expression had a significantly lower cumulative 5-year overall survival rate than those with negative expression (28.3% *vs* 51.9%, $P < 0.0001$). Among the 99 patients with stage Ⅲ GC, those with positive PRL-3 expression had a lower survival rate than those with negative expression (18.6% *vs* 43.2%, $P = 0.0004$, Figure 2). Among the 200 patients who received curative surgery, patients whose tumor had positive PRL-3 expression had worse disease-free status and poorer overall survival (hazard ratio, 16.6 and 16.7 respectively; $P < 0.0001$ for both) than those with negative expression (Figure 3). Among patients who received palliative resection or patients in stages other than stage Ⅲ, PRL-3 showed no significant correlation with prognosis.

Multivariate survival analysis of prognostic impact of PRL-3 expression

Multivariate analysis by extended Cox regression model revealed that PRL-3 expression remained an independent prognostic factor after adjusting for sex, age, tumor location, tumor size, depth of invasion, lymph node metastasis, distant metastasis, TNM staging, vascular/lymphatic invasion, and surgical curability. PRL-3 expression was a significantly independent prognostic factor for the overall survival of all 293 GC patients. For the 200 patients who received curative resection, PRL-3 expression was found to be an independent prognostic factor for both disease-free and overall survival. The results are shown in Table 2.

DISCUSSION

In this study, we detected the protein expression

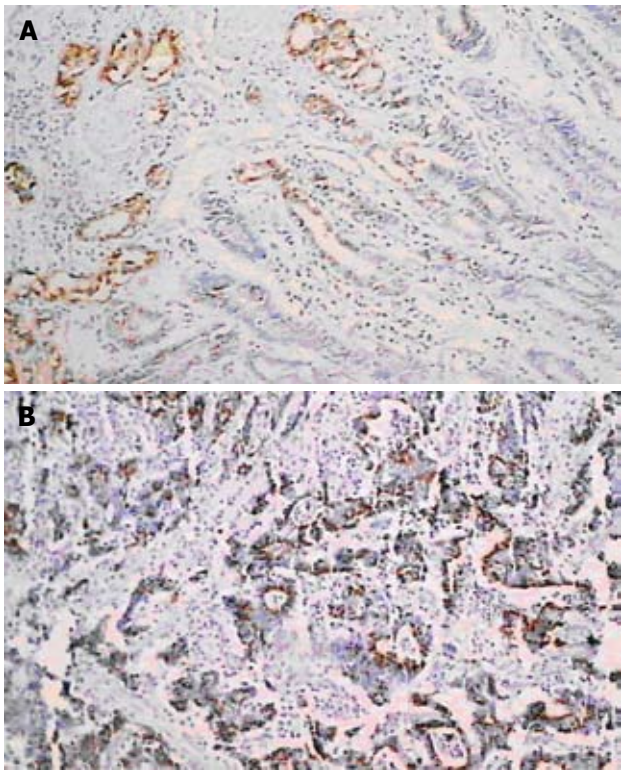

Figure 1 Immunohistochemical staining. A: PRL-3 is negative or weak in adjacent (3 cm away from the tumor) normal gastric epithelial mucosa (× 40); B: In positive cases, PRL-3 expression in cancer cell cytoplasms is strong (× 200).

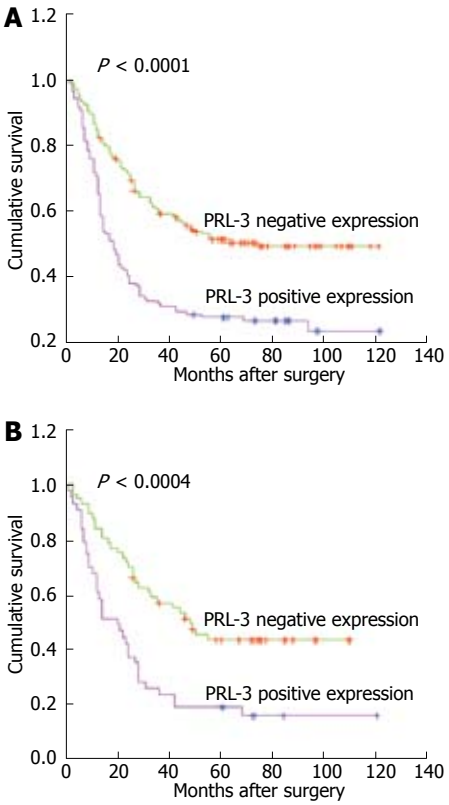

Figure 2 Overall survival curve. A: Entire cohort of 293 patients; B: Patients with stage Ⅲ. Significant differences were observed between the two groups with PRL-3 negative and positive expression.

of PRL-3 in GC tissues using the highly specific monoclonal antibody 3B6 prepared by Peng *et al*. PRL-3

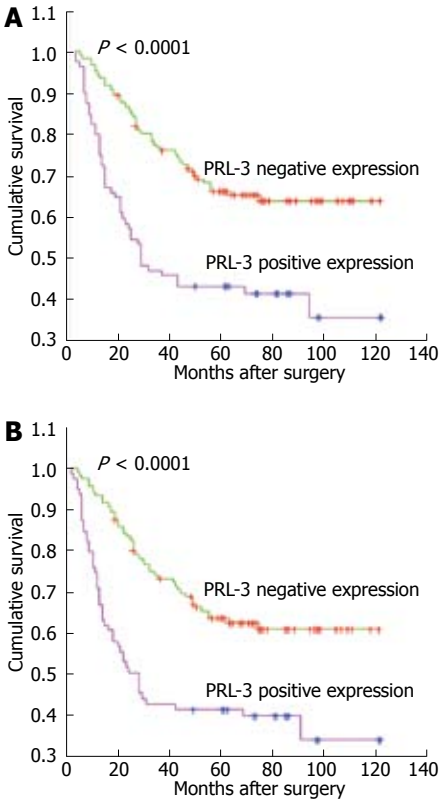

Figure 3 Patients who underwent curative surgery. A: Overall survival; B: Disease-free survival. Significant differences were observed between the PRL-3 negative and positive groups.

had higher rates of positive expression in advanced stages and PRL-3 expression was positively correlated with tumor size, depth of invasion, and lymph node metastasis vascular/lymphatic invasion at the time of surgery and recurrence. These results suggest that PRL-3 may play a crucial role in invasion, progression and metastasis of GC. The present analyses revealed that PRL-3 was an independent prognostic indicator for overall and disease-free survival of GC. Among patients with advanced TNM stages especially stage Ⅲ, patients with positive PRL-3 expression have more frequent recurrence and poorer survival, adjuvant therapies such as radiotherapy and chemotherapy may be necessary after curative surgery. Evaluation of PRL-3 expression status may identify a subset of patients with GC who require more intensive treatment.

Miskad *et al*[20] reported that PRL-3 was highly expressed in metastatic lymph nodes of GC and high expression of PRL-3 was closely associated with tumor stage. Wang *et al*[21] found that the high expression of PRL-3 in lymph node metastases had a negative impact on the prognosis of patients with GC. Li *et al*[22-23] reported that PRL-3 expression was correlated with peritoneal metastasis and poor prognosis in GC patients. The superiority of our study may be the use of antibody specifically against PRL-3 and the relatively extensive clinical data which facilitated the analysis from multiple angels. One limitation of our study is that the relatively small sample hindered the analysis in stage Ⅰ and stage Ⅱ patients.

Table 2 Multivariate analysis of PRL-3 expression by Cox proportional hazard model

Factors	Overall survival						Disease-free survival		
	All patients (*n* = cases)			Curatively resected (*n* = 200)			Curatively resected (*n* = 200)		
	P	Relative risk	95% CI	*P*	Relative risk	95% CI	*P*	Relative risk	95% CI
PRL-3	< 0.001	1.76	1.30-2.40	< 0.001	2.35	1.54-3.59	< 0.001	2.46	1.62-3.73
T	< 0.001	3.16	1.79-5.58	0.003	3.04	1.47-6.29	0.004	2.76	1.38-5.52
N	< 0.001	3.29	1.93-5.60	< 0.001	3.99	2.21-7.20	< 0.001	4.67	2.59-8.43
D	0.04	1.38	1.01-1.88	0.32	1.23	0.82-1.87	0.015	1.69	1.11-2.57
S	< 0.001	2.24	1.54-3.26	-	-	-	-	-	-

PRL-3 expression, positive *vs* negative; T: Depth of invasion, T3-4 *vs* T1-2; N: Lymph node metastasis, present *vs* absent; D: Tumor size, ⩾ 5 cm *vs* < 5 cm; S: Surgical curability, palliative *vs* curative.

Attributed to the high sequence similarity of three PRLs and the wide expression of PRL-1 and PRL-2 in normal tissues and cancer cell lines, commercial polyclonal antibody against PRL-3 used in previous studies could potentially cross-react with PRL-1 and PRL-2. Monoclonal antibody specifically reacting with PRL-3 is extremely important to exclude the interference of PRL-1 and PRL-2 and therefore allows us to accurately evaluate the prognostic implication of PRL-3 expression[25,26]. To prepare specific PRL-3 monoclonal antibody, Peng *et al*[25] obtained the monoclonal antibody 3B6 with hybridoma technique, and confirmed its specificity with ELISA and Western blotting assays. High specificity of the monoclonal antibody 3B6 against PRL-3 was demonstrated. The applicability of the monoclonal antibody has been further confirmed by two other studies investigating the prognostic impact of PRL-3 expression in colorectal cancer and breast cancer[16,19].

PRL-3 has been confirmed to be an important metastatic instrumental molecule. Although the actual signal transduction pathways in which PRL-3 is implicated are largely unknown, Rho signaling pathway molecules which are regulators of motility and invasion have been identified as potential candidate targets of PRL-3. PRL-3 transfectants displayed altered extracellular matrix adhesive property and up-regulated integrin-mediated cell spreading efficiency[14,27]. Peng *et al*[28] recently found that PRL-3 activates the mitogen-associated protein kinase pathway by binding a cell membrane protein in cell migration and invasion. PRL-3 was also found to be associated with membrane structures including ruffles, protrusions, and some vacuolar-like membrane extensions which have been demonstrated to play a role in cell movement and invasion[29-31]. Besides, PRL-3 may be involved in triggering angiogenesis and establishing microvasculature *in vitro*[32-34]. These findings suggest that PRL-3 may be implicated with the key steps of tumor metastasis including tumor cell invasion and survival in circulation and vasculature formation.

In addition to its role in predicting tumor recurrence and prognosis, PRL-3 has a potential value of being a candidate for metastasis tailored therapies. Since primary tumors can be surgically resected, the metastatic tumors are the main cause responsible for a high case fatality. PRL-3 was highly expressed in tumor metastasis and found to play a key role in tumor metastatic process[10-21]. PRL-3 may serve as a potential therapeutic target for cancer metastases. Inhibition of PRL activity might be carried out using phosphatase inhibitors targeting the consensus phosphatase motif, farnesyltransferase inhibitors, interference RNA or monoclonal antibody as well[9,35-37]. Recent progress in active recombinant PRL-3 production and findings on PRL-3 structure will undoubtedly facilitate the development of PRL-3 inhibitors[38-40]. Detection of PRL-3 expression would be able to provide supportive information for anti-cancer therapy.

In conclusion, PRL-3 is closely associated with tumor invasion and lymphatic metastasis and is identified as a new prognostic indicator to predict tumor recurrence and patient survival in GC.

COMMENTS

Background

It is established that phosphatase regenerating liver 3 (PRL-3) is consistently expressed in liver metastasis of colon cancer. A recent study reported that PRL-3 expression was related to peritoneal metastasis of stomach cancer.

Research frontiers

In a few studies, the association of PRL-3 expression with prognosis of cancers was investigated and demonstrated that this is related to poor prognosis of breast and stomach cancer. The antibody used in many studies was commercial polyclonal antibody that could cross-react with PRL-1 and PRL-2.

Innovations and breakthroughs

A recent study showed that PRL-3 was expressed in 70.4% of 637 GCs. PRL-3 expression was correlated with peritoneal metastasis. Patients with PRL-3 negative expression had a better survival rate than those with positive PRL-3 at all stages. In this study, PRL-3 was found to express in 43.3% (127/293) of primary tumor tissues of GC. PRL-3 protein expression was demonstrated to be an independent predictor for poor prognosis in GC. In addition, a specific monoclonal antibody to PRL-3 was used, allowing us to accurately evaluate the prognostic significance of PRL-3 expression in GC.

Applications

Defection of PRL-3 protein expression in primary tumor tissues of GC might be helpful in identification of GC patients with poor prognosis who should receive more intensive treatment. In addition, PRL-3 may serve as a potential therapeutic target for GC.

Terminology

PRL-3 (also known as PTP4A3) is a member of phosphate of regeneration liver (PRL) family including PRL-1, PRL-2 and PRL-3 which are implicated with oncogenic and metastatic processes of tumors. The excess of PRL-3 phosphates is an alteration contributing to the acquisition of metastatic properties of tumor cells. The monoclonal antibody 3B6 used in this study is a specific monoclonal antibody to PRL-3 generated by Peng *at al*, Beijing Institute for Cancer Research.

Peer review

The authors examined the expression of PRL-3 protein in primary tumor tissues of 293 cases of GC. It revealed that patients with negative PRL-3 expression had a higher 5-year survival rate than those with positive PRL-3 expression. PRL-3 is an independent prognostic indicator for GC. The findings are of clinical significance.

REFERENCES

1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

2 **Maehara Y**, Oshiro T, Endo K, Baba H, Oda S, Ichiyoshi Y, Kohnoe S, Sugimachi K. Clinical significance of occult micrometastasis lymph nodes from patients with early gastric cancer who died of recurrence. *Surgery* 1996; **119**: 397-402

3 **Diamond RH**, Cressman DE, Laz TM, Abrams CS, Taub R. PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. *Mol Cell Biol* 1994; **14**: 3752-3762

4 **Wang Q**, Holmes DI, Powell SM, Lu QL, Waxman J. Analysis of stromal-epithelial interactions in prostate cancer identifies PTPCAAX2 as a potential oncogene. *Cancer Lett* 2002; **175**: 63-69

5 **Cates CA**, Michael RL, Stayrook KR, Harvey KA, Burke YD, Randall SK, Crowell PL, Crowell DN. Prenylation of oncogenic human PTP(CAAX) protein tyrosine phosphatases. *Cancer Lett* 1996; **110**: 49-55

6 **Zeng Q**, Hong W, Tan YH. Mouse PRL-2 and PRL-3, two potentially prenylated protein tyrosine phosphatases homologous to PRL-1. *Biochem Biophys Res Commun* 1998; **244**: 421-427

7 **Dumaual CM**, Sandusky GE, Crowell PL, Randall SK. Cellular localization of PRL-1 and PRL-2 gene expression in normal adult human tissues. *J Histochem Cytochem* 2006; **54**: 1401-1412

8 **Matter WF**, Estridge T, Zhang C, Belagaje R, Stancato L, Dixon J, Johnson B, Bloem L, Pickard T, Donaghue M, Acton S, Jeyaseelan R, Kadambi V, Vlahos CJ. Role of PRL-3, a human muscle-specific tyrosine phosphatase, in angiotensin-II signaling. *Biochem Biophys Res Commun* 2001; **283**: 1061-1068

9 **Zeng Q**, Si X, Horstmann H, Xu Y, Hong W, Pallen CJ. Prenylation-dependent association of protein-tyrosine phosphatases PRL-1, -2, and -3 with the plasma membrane and the early endosome. *J Biol Chem* 2000; **275**: 21444-21452

10 **Saha S**, Bardelli A, Buckhaults P, Velculescu VE, Rago C, St Croix B, Romans KE, Choti MA, Lengauer C, Kinzler KW, Vogelstein B. A phosphatase associated with metastasis of colorectal cancer. *Science* 2001; **294**: 1343-1346

11 **Bardelli A**, Saha S, Sager JA, Romans KE, Xin B, Markowitz SD, Lengauer C, Velculescu VE, Kinzler KW, Vogelstein B. PRL-3 expression in metastatic cancers. *Clin Cancer Res* 2003; **9**: 5607-5615

12 **Zeng Q**, Dong JM, Guo K, Li J, Tan HX, Koh V, Pallen CJ, Manser E, Hong W. PRL-3 and PRL-1 promote cell migration, invasion, and metastasis. *Cancer Res* 2003; **63**: 2716-2722

13 **Guo K**, Li J, Tang JP, Koh V, Gan BQ, Zeng Q. Catalytic domain of PRL-3 plays an essential role in tumor metastasis: formation of PRL-3 tumors inside the blood vessels. *Cancer Biol Ther* 2004; **3**: 945-951

14 **Molleví DG**, Aytes A, Padullés L, Martínez-Iniesta M, Baixeras N, Salazar R, Ramos E, Figueras J, Capella G, Villanueva A. PRL-3 is essentially overexpressed in primary colorectal tumours and associates with tumour aggressiveness. *Br J Cancer* 2008; **99**: 1718-1725

15 **Stephens BJ**, Han H, Gokhale V, Von Hoff DD. PRL phosphatases as potential molecular targets in cancer. *Mol Cancer Ther* 2005; **4**: 1653-1661

16 **Peng L**, Ning J, Meng L, Shou C. The association of the expression level of protein tyrosine phosphatase PRL-3 protein with liver metastasis and prognosis of patients with colorectal cancer. *J Cancer Res Clin Oncol* 2004; **130**: 521-526

17 **Kato H**, Semba S, Miskad UA, Seo Y, Kasuga M, Yokozaki H. High expression of PRL-3 promotes cancer cell motility and liver metastasis in human colorectal cancer: a predictive molecular marker of metachronous liver and lung metastases. *Clin Cancer Res* 2004; **10**: 7318-7328

18 **Radke I**, Götte M, Kersting C, Mattsson B, Kiesel L, Wülfing P. Expression and prognostic impact of the protein tyrosine phosphatases PRL-1, PRL-2, and PRL-3 in breast cancer. *Br J Cancer* 2006; **95**: 347-354

19 **Wang L**, Peng L, Dong B, Kong L, Meng L, Yan L, Xie Y, Shou C. Overexpression of phosphatase of regenerating liver-3 in breast cancer: association with a poor clinical outcome. *Ann Oncol* 2006; **17**: 1517-1522

20 **Miskad UA**, Semba S, Kato H, Yokozaki H. Expression of PRL-3 phosphatase in human gastric carcinomas: close correlation with invasion and metastasis. *Pathobiology* 2004; **71**: 176-184

21 **Wang Z**, He YL, Cai SR, Zhan WH, Li ZR, Zhu BH, Chen CQ, Ma JP, Chen ZX, Li W, Zhang LJ. Expression and prognostic impact of PRL-3 in lymph node metastasis of gastric cancer: its molecular mechanism was investigated using artificial microRNA interference. *Int J Cancer* 2008; **123**: 1439-1447

22 **Li Z**, Zhan W, Wang Z, Zhu B, He Y, Peng J, Cai S, Ma J. Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. *Biochem Biophys Res Commun* 2006; **348**: 229-237

23 **Li ZR**, Wang Z, Zhu BH, He YL, Peng JS, Cai SR, Ma JP, Zhan WH. Association of tyrosine PRL-3 phosphatase protein expression with peritoneal metastasis of gastric carcinoma and prognosis. *Surg Today* 2007; **37**: 646-651

24 **Sobin LH**, Fleming ID. TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997; **80**: 1803-1804

25 **Peng L**, Li Y, Meng L, Shou C. Preparation and characterization of monoclonal antibody against protein tyrosine phosphatase PRL-3. *Hybrid Hybridomics* 2004; **23**: 23-27

26 **Li J**, Guo K, Koh VW, Tang JP, Gan BQ, Shi H, Li HX, Zeng Q. Generation of PRL-3- and PRL-1-specific monoclonal antibodies as potential diagnostic markers for cancer metastases. *Clin Cancer Res* 2005; **11**: 2195-2204

27 **Fiordalisi JJ**, Keller PJ, Cox AD. PRL tyrosine phosphatases regulate rho family GTPases to promote invasion and motility. *Cancer Res* 2006; **66**: 3153-3161

28 **Peng L**, Jin G, Wang L, Guo J, Meng L, Shou C. Identification of integrin alpha1 as an interacting protein of protein tyrosine phosphatase PRL-3. *Biochem Biophys Res Commun* 2006; **342**: 179-183

29 **Chambers AF**, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002; **2**: 563-572

30 **Nobes CD**, Hall A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* 1999; **144**: 1235-1244

31 **Small JV**, Stradal T, Vignal E, Rottner K. The lamellipodium: where motility begins. *Trends Cell Biol* 2002; **12**: 112-120

32 **Guo K**, Li J, Wang H, Osato M, Tang JP, Quah SY, Gan BQ, Zeng Q. PRL-3 initiates tumor angiogenesis by recruiting endothelial cells in vitro and in vivo. *Cancer Res* 2006; **66**: 9625-9635

33 **Parker BS**, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S, Madden SL. Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* 2004; **64**: 7857-7866

34 **St Croix B**, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Genes expressed in human tumor endothelium.

Science 2000; **289**: 1197-1202
35 **Pathak MK**, Dhawan D, Lindner DJ, Borden EC, Farver C, Yi T. Pentamidine is an inhibitor of PRL phosphatases with anticancer activity. *Mol Cancer Ther* 2002; **1**: 1255-1264
36 **Sebti SM**, Der CJ. Opinion: Searching for the elusive targets of farnesyltransferase inhibitors. *Nat Rev Cancer* 2003; **3**: 945-951
37 **Guo K**, Tang JP, Tan CP, Wang H, Zeng Q. Monoclonal antibodies target intracellular PRL phosphatases to inhibit cancer metastases in mice. *Cancer Biol Ther* 2008; **7**: 750-757
38 **Sager JA**, Benvenuti S, Bardelli A. PRL-3: a phosphatase for metastasis? *Cancer Biol Ther* 2004; **3**: 952-953
39 **Kim KA**, Song JS, Jee J, Sheen MR, Lee C, Lee TG, Ro S, Cho JM, Lee W, Yamazaki T, Jeon YH, Cheong C. Structure of human PRL-3, the phosphatase associated with cancer metastasis. *FEBS Lett* 2004; **565**: 181-187
40 **Kozlov G**, Cheng J, Ziomek E, Banville D, Gehring K, Ekiel I. Structural insights into molecular function of the metastasis-associated phosphatase PRL-3. *J Biol Chem* 2004; **279**: 11882-11889

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BRIEF ARTICLES

Intrahepatic transplantation of hepatic oval cells for fulminant hepatic failure in rats

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Abstract

AIM: To evaluate the effect of intrahepatic transplantation of hepatic oval cells (HOC) on fulminant hepatic failure (FHF) in rats.

METHODS: HOC obtained from rats were labeled with green fluocescent protein (GFP) or 5, 6-carboxyfluorescein diacetate succinmidyl ester (CFDA-SE). Cell fluorescence was observed under fluorescent microscope at 6, 24, 48 and 72 h after labeling. CFDA-SE labeled HOC (5×10^6 cells each rat) were injected into livers of rats with FHF induced by D-galactosamine. Serum albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels were measured at different time points. Liver function of rats was examined on days 3, 7, 14 and 21 after HOC transplantation.

RESULTS: The positive rate of GFP and CFDA-SE labeled HOC was 10% and 90%, respectively, with no significant change in cell viabilities. The survival rate was higher in HOC transplantation group than in control group, especially 48 (9/15 *vs* 6/15) and 72 h (9/15 *vs* 4/15) after HOC transplantation. The serum ALT, AST and TBil levels were decreased while the serum Alb level was increased after HOC transplantation. Fluorescence became faded and diffused in liver tissues, suggesting that proliferation and differentiation occur in transplanted HOC.

CONCLUSION: CFDA-SE is superior to GFP in labeling HOC, although fluorescence intensity is decreased progressively with cell division. HOC transplantation can improve the liver function and increase the survival rate of recipients.

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Key words: Liver; Stem cells; Hepatic oval cells; Fluorescence labeling; Transplantation; Fulminant hepatic failure

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INTRODUCTION

Hepatic oval cells (HOC) are liver stem cells with a self-renewal capacity and a high proliferation potential[1]. Transplantation of HOC cultured *in vitro* can restore damaged liver function, thus providing more opportunities for patients with terminal-stage liver diseases[2,3]. In this study, we established a rat HOC proliferation model by feeding 2-acetylaminofluorie (2-AAF) and resecting 2/3 liver. HOC were isolated, purified and labeled with 5, 6-carboxyfluorescein diacetate succinmidyl ester (CFDA-SE), a fluorescence agent, before they were transplanted into the rats with fulminant hepatic failure (FHF). Then, we detected the fluorescence distribution in the recepient liver and a few laboratory indexes, trying to find the effect of HOC transplantation on FHF.

MATERIALS AND METHODS

Animals and reagents

Wistar rats were provided by the Animal Experimental Center of the Radiation Institute, Chinese Academy of

Sciences (Beijing, China). 2-AAF was purchased from Sigma and D-galactosamine (D-GalN) was provided by Chongqing Medical University (Chongqing, China). *E. coli* strain harboring plasmids carrying the GFP gene, Pmax-GFP, was produced by Amaxa. Fugene HD transfection regent was from Rocheand. CFDA-SE was from Molecular Probes.

Establishment of a rat HOC proliferation model

Twenty healthy Wistar rats, weighing 180-220 g, received intra-gastric 2-AAF, 15 mg/kg per day, for 4 d. On day 5, the rats were anesthetized with 1% sodium pentobarbital and their left and middle liver lobes (about 2/3 of the liver volume) were resected. From day 6, the rats were given 2-AAF, 15 mg/kg per day, for additional 10 d to induce a rat HOC proliferation.

Isolation, cultivation and identification of HOC

Hepatic cells were separated from the rat model by the improved *in situ* perfusion of Seglen collagenase[4]. HOC were purified from the separated hepatic cells by Percoll density gradient centrifugation and inoculated in a DMEM/F12 culture medium at a concentration of 1×10^6/mL. The cells were cultured at 37℃ in an atmosphere containing 50 mL/L CO_2, and half of the medium was changed every two days. Cell morphology and expansion were regularly observed under an inverted microscope. The cells were passaged when necessary and observed under an electronic microscope. OV-6, AFP, ABL and PCNA, expressed on cells were tested by immunohistochemical assay.

Fugene HD-mediated transfection of HOC with the GFP gene

HOC were transfected with Fugene HD transfection regent following its manufacturer's instructions. Briefly, HOC at passage 1 were seeded onto 12-well plates at a density of 2×10^5/mL. One day later, transfection compounds at different ratios [transfection agent (μL): plasmid (μg) = 3:2, 4:2, 5:2, 6:2, 7:2 and 8:2] were added to the culture medium and shaken for 30 s at a low speed to ensure a homogeneous mixture. Then, the cells were incubated at 37℃ in a humidified atmosphere containing 50 mL/L CO_2. After 6, 12, 24, 48 and 72 h, samples were taken from three random sections in each well and observed under an inverted confocal microscope and 100 cells were counted. GFP expression in these cells was observed under a fluorescence microscope with the excitation wavelength at 488 nm and the emission wavelength at 507 nm. The transfection rate was calculated according to the following equation: transfection rate (%) = number of green fluorescent cells in dark field/number of cells in bright field. The cell proliferation was determined by 3-(4,5-dimethylthiazolzyl)-2,5-diphenyltetrazolium bromide (MTT) colorimetry and growth curves were plotted.

HOC labeling by fluorochrome CFDA-SE

Passage 1 HOC, reaching an approximate confluence of 80%, were adjusted to 1×10^6/mL in serum-free PBS with 5 μmol/L CFDA-SE and incubated at 37℃ for 10 min. After the same volume of complete medium was added to terminate the staining, cells were separated by centrifugation and the staining was repeated three times before incubation. After 0, 6, 24 and 72 h, the cells were observed under a fluorescence microscope at 488 nm. Cell growth activity was also determined by MTT colorimetry and growth curves were plotted.

HOC transplantation for FHF in rats

Thirty Wistar rats were intra-peritoneally injected with a 10% D-GalN solution at a dose of 1400 mg/kg to induce FHF. One day after FHF induction, rats were divided into transplantation group (n = 15) and control group (n = 15). Rats in HOC proliferation model were anaesthetized at the supine position, and a 1.5 cm incision was made at the middle of the upper abdomen to expose the liver. The number of fluorescence labeled HOC was adjusted to 1×10^7/mL for transplantation. Rats in the transplant group were injected with 0.5 mL CFDA-SE labeled HOC suspension in the left lobe of liver, while rats in the control group were given the same volume of culture medium. After 1, 2, 3, 5 and 7 d of transplantation, blood sample was taken from the rat tail and liver function was determined with an automatic biochemical analyzer. Albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels were measured. After 3, 7, 14 and 21 d, the animals were killed and their livers were removed for pathological examination. Frozen liver tissue around the injected site was cut into sections to observe the distribution of fluorescence labeled cells in the liver tissue under a fluorescent microscope.

Statistical methods

All data were analyzed by SPSS 13.0. Two sets of sample means (mean ± SD) were compared by t-test and the percentages were compared by χ^2-test. $P < 0.05$ was considered statistically significant.

RESULTS

HOC incubation

The freshly separated rat HOC adhered to the wall after 12 h of incubation were spindle or polygonal in shape. After about 7 d, the cells grew into colonies. The HOC, 10 d after passage, grew into a single-layer flagstone which did not change 14 d after passage (Figure 1).

Morphological and phenotypic identification of the cells

The expression of OV-6, AFP and ALB in HOC was detected by immunohistochemistry. Positive staining of OV-6 and AFP was detected in isolated HOC while no ALB expression was found in HOC. Electron microscopy showed short and tiny microvilli-like protuberances on the surface of HOC. The cell nuclei were oval with dispersed and homogenous nuclear chromatin, small nucleoli, little cytoplasm, great nucleus-cytoplasm ratio

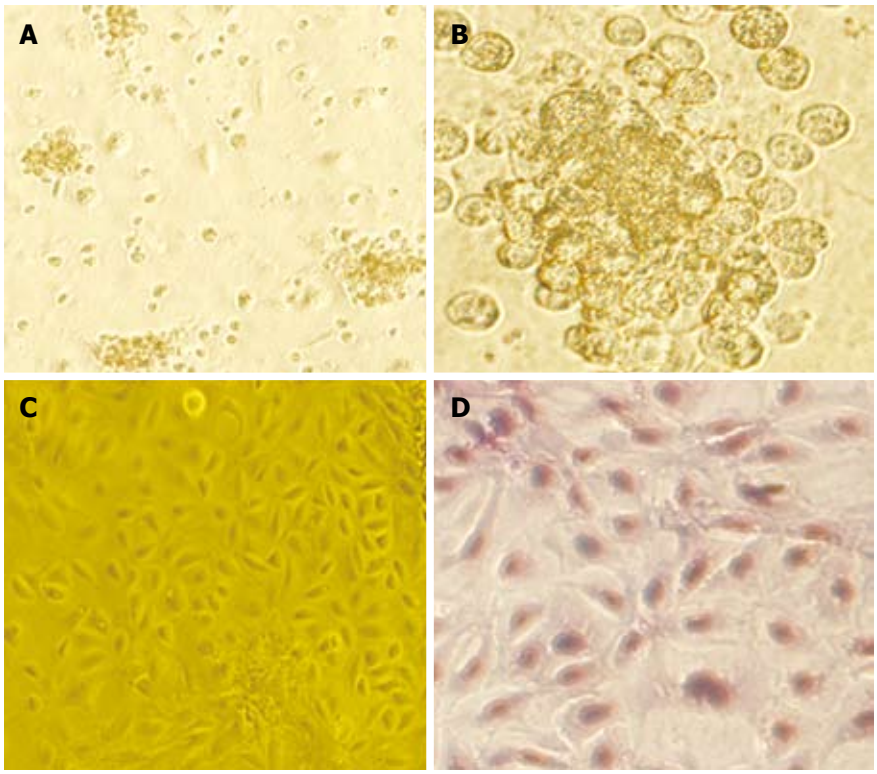

Figure 1 HE stained HOC 10 h (A, × 100), 7 (B, × 400), 10 (C, × 100), and 14 d (D, × 200) after incubation.

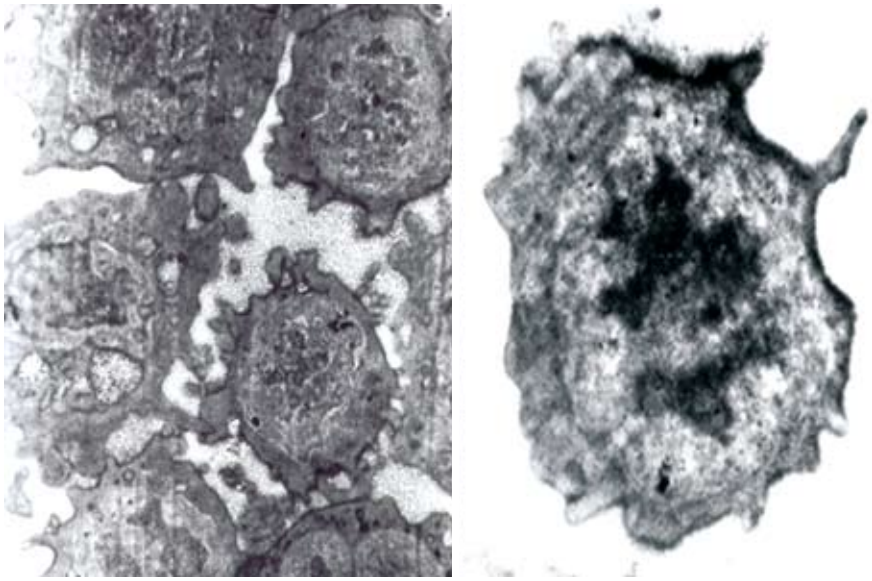

Figure 2 Ultra-structure of rat HOC under electron microscope (× 4000).

and underdeveloped endoplasmic reticulum, mitochondria and ribosome (Figure 2), indicating that incubated HOC are primitive, naive and undifferentiated.

HOC labeling

Six hours after transfection with the GFP gene, a low GFP expression level in some HOC could be observed under a fluorescent microscope. The GFP gene was expressed both in nuclei and in cytoplasm. Its expression increased significantly after 24 h, reached its peak at 48 h and maintained till 72 h. A higher transfection efficiency (about 10.0%) could be achieved at a transfection reagent-plasmid ratio of 5:2. The fluorescent intensity of transfected cells was gradually reduced and disappeared after 5-7 generations. Green fluorescence could be observed immediately after CFDA-SE labeling, with a labeling rate of 90%. The fluorescence intensity decreased slightly after 6 h and significantly after 24 h. However, the fluorescence intensity was almost the same at 72 h and 24 h (Figure 3).

Liver functions and survival of FHF rats after HOC transplantation

The rats in HOC transplantation group slightly restored their general conditions, food taking and movements 48 h after transplantation. On the contrary, the conditions of most rats in control group were further exacerbated. The serum ALB, AST, ALT and TBiL levels in rats of both groups are listed in Table 1. Death occurred in rats of both groups around 6 h after transplantation.

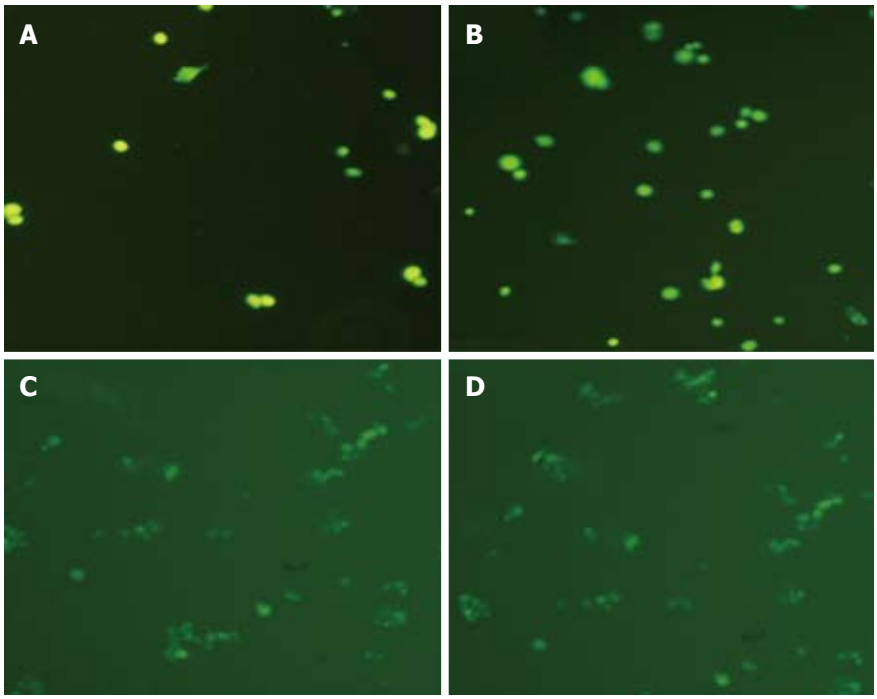

Figure 3 CFDA-SE stained HOC at 0 h (A, × 200), 6 h (B, × 200), 24 h (C, × 100), and 72 h (D, × 100).

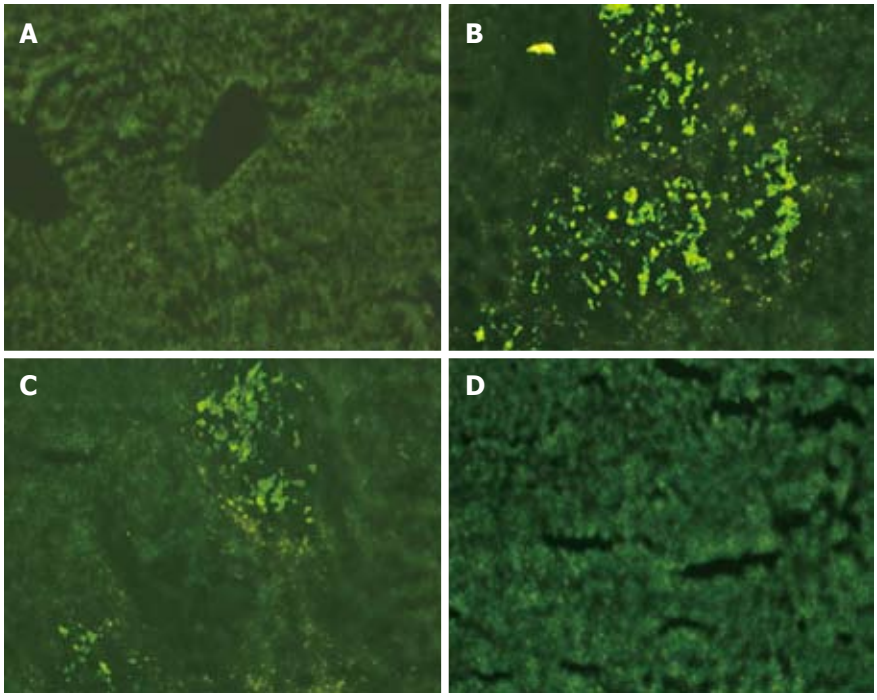

Figure 4 Fluorescent microscopy showing colonies in control group (A), and 3 (B), 7 (C) and 14 d (D) after HOC transplantation (× 100).

However, no rat died in transplantation group 72 h after transplantation. The survival rate for rats in transplantation group and control group was 60% (9/15) and 26.7% (4/15), respectively.

Fluorescent microscopy after HOC transplantation

Green fluorescent cell colonies ccould be seen in sections of frozen rat liver tissue three days after transplantation. Most fluorescent colonies were located at the injection site with a strong fluorescent intensity. The fluorescent intensity decreased seven days after transplantation but the number of colonies increased with a wider distribution. No fluorescent cell was detected in the liver sample 14 d after HOC transplantation and afterwards (Figure 4).

DISCUSSION

The Solt-Farber model[4] is the most commonly used model for HOC proliferation, and has been used in studying the relationship between local disease and liver cancer or between nodules and liver cancer. Collagenase perfusion, proposed by Howard *et al*[5] and improved by Berry and Seglen *et al*[6,7], is often used in detection of HOC separation. In this study, Solt-Farber model was used to detect HOC proliferation, and a large

Table 1 Changes of ALB, ALT, AST and TBilL levels in rat liver tissue after HOC transplantation (mean ± SD)

	Groups (survival)	ALB (g/L)	ALT (U/L)	AST (U/L)	TBilL (μmol/L)
Transplantation 0 d	Control (15)	14.6 ± 1.9	757.3 ± 47.2	348.0 ± 66.5	55.4 ± 7.1
	Transplantation (15)	15.2 ± 2.4	736.3 ± 58.1	357.4 ± 42.3	50.6 ± 4.6
1 d after transplantation	Control (10)	13.4 ± 2.5	789.6 ± 27.5	384.6 ± 73.3	56.6 ± 7.1
	Transplantation (10)	14.2 ± 1.8[a]	803.3 ± 62.4[a]	375.3 ± 49.2[a]	61.6 ± 19.2[a]
2 d after transplantation	Control (6)	11.6 ± 1.6	873.5 ± 43.2	409.0 ± 31.8	60.3 ± 6.5
	Transplantation (9)	20.3 ± 1.3[b]	649.0 ± 90.3[b]	263.3 ± 28.2[b]	53.0 ± 4.2[a]
3 d after transplantation	Control (4)	9.8 ± 0.6	896.6 ± 44.8	434.3 ± 25.4	46.3 ± 3.7
	Transplantation (9)	26.3 ± 0.9[b]	430.0 ± 28.3[b]	124.6 ± 21.6[b]	23.7 ± 6.9[b]
5 d after transplantation	Control (3)	13.5 ± 1.2	774.6 ± 26.7	326.6 ± 15.5	45.8 ± 4.3
	Transplantation (8)	27.8 ± 2.6[b]	377.3 ± 29.4[b]	106.0 ± 15.3[b]	19.5 ± 5.2[b]
7 d after transplantation	Control (3)	19.7 ± 1.6	564.2 ± 43.2	246.3 ± 26.7	32.3 ± 5.0
	Transplantation (8)	31.5 ± 2.6[b]	333.3 ± 36.4[b]	89.3 ± 13.2[b]	6.9 ± 1.8[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

amount of small proliferation focuses were found in portal area. The HOC were oval or oblong in shape, and their size was much smaller than that of hepatic cells (about 1/6-1/3 of the normal size). Moreover, immunohistochemical staining showed positive OV-6 and AFP expression, consistent with the traits of HOC. The positive PCNA staining showed that HOC were at their proliferating stage and that HOC proliferation in liver of adult rats could be induced by 2-AAF injection and 2/3 liver resection. After the proliferation model was established, suspended hepatic cells were prepared by two-step collagenase perfusion. The cells were purified by density gradient centrifugation and observed under an electron microscope. The purified cells were primitive, naive and undifferentiated. Immunohistochemical staining of OV-6 and AFP in the freshly separated cells was similar to that of proliferated cells in the model. The cells showed a certain proliferation capacity in culture and were heterogenous as previously reported[8-10].

The therapeutic effect of HOC transplantation on FHF has been proved both in animal models and in clinical trials[11,12]. Matsusaka *et al*[13] transplanted hepatic cells with a large number of HOC into the spleen of rats, and found that hepatic cells can significantly proliferate compared to those without HOC. Yasui *et al*[14] transplanted HOC into the liver of Nagase rats (a family of rats with inherited serum albumin deficiency), and showed that the serum albumin level maintained high in these rats for 10 wk, indicating that HOC have differentiated into mature and functional hepatic cells. In this study, the rat FHF model was induced by D-GalN, into which rat HOC were transplanted. Biochemical assay showed the liver functions and pathological lesions of rats were slightly improved 48 h after transplantation. Moreover, the ALB and ALT levels were decreased in the following days, indicating that the transplanted HOC can survive in rats with FHF, and proliferate and differentiate to replace the damaged hepatic cells. The effect of HOC transplantation on FHF is related to the strong proliferation and differentiation of HOC into mature hepatic cells and biliary epithelial cells, which consequently benefit rat survival. In addition, liver failure elicits liver regeneration and up regulation of hepatocyte growth factors. These cytokines, forming a suitable microenvironment, are necessory for the survival, growth and proliferation of transplanted HOC. Thus, newly regenerated hepatocytes compensate the damaged liver function, their robust activity may interact with adjacent cells and rescue some damaged liver cells with reversible pathologic lesions.

It is essential to appropriately label the transplanted cells to track their location and function in receptor. Both GFP and CFDAS-SE are fluorescent labels for *in vivo* cell transplantation, but CFDA-SE showed superior properties in this study. CFDA-SE, a fluorescent dye, has been applied in various fields of immunology due to its stability and long duration. When cells divide, CFDA-SE is equally divided into two daughter cells, leading to an exponential decrease in fluorescence intensity with cell proliferation and division[15]. In this study, CFDA-SE labeled HOC were transplanted in rats with FHF. Seventy-two hours after transplantation, green fluorescent colonies could be observed in sections of frozen liver tissue. The fluorescence intensity was strong but the colonies were only found near the injection site. On day 7, the fluorescence intensity of the transplanted cells decreased but transplanted cells were widely distributed, indicating that HOC have proliferated and differentiated into hepatic cells. Therefore, multiple green fluorescent colonies could be observed.

In conclusion, labeled HOC transplantation exerts its effects on FHF by improving the serum levels of ALT, AST, and TBil. However, since fluorescence intensity of CFDA-SE decreases with cell division, it is still not the ideal label for cell transplantation. Further study is needed on the location and distribution of transplanted HOC.

COMMENTS

Background

Fulminant hepatic failure is a serious clinical disease and may threaten the life of patients. However, because of the damage of mass liver cells, the organ function is often irreversible due to the liver cell degeneration, swelling, or apoptosis. Thus, to supply new sources of functional liver cells is a valuable choice for these patients.

Innovations and breakthroughs

The cultured hepatic oval cells (HOC) can provide cells for liver cell transplantation and even for biological artificial liver, thus solving the problem of liver donor shortage. In this study, a rat HOC proliferation model was established and the HOC were isolated, purified, labeled with CFDA-SE (a fluorescence agent), and transplanted into rats with fulminant hepatic failure (FHF). Then the authors detected the fluorescence distribution in the receptor liver to observe the role of HOC transplantation in FHF treatment.

Applications

The study indicated that transplantation of hepatic oval cells was a potential therapeutic strategy for the treatment of fulminant hepatic failure.

Terminology

HOC: liver stem cells with a self-renewal capacity and a high proliferative potential. FHF is usually defined as the severe impairment of hepatic functions in the absence of preexisting liver disease. 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE): a fluorescence agent

Peer review

The manuscript reports a therapeutic potential of transplantation of hepatic oval cells for fulminant hepatitis. Although liver transplantation is the most effective therapy for fulminant hepatitis at present, cell-based therapy could be an alternative treatment modality. The data presented are encouraging and promising.

REFERENCES

1 **Braun KM**, Thompson AW, Sandgren EP. Hepatic microenvironment affects oval cell localization in albumin-urokinase-type plasminogen activator transgenic mice. *Am J Pathol* 2003; **162**: 195-202

2 **Forbes S**, Vig P, Poulsom R, Thomas H, Alison M. Hepatic stem cells. *J Pathol* 2002; **197**: 510-518

3 **Stieger B**, Peters R, Sidler MA, Meier PJ. Hepatocyte transplantation: potential of hepatocyte progenitor cells and bone marrow derived stem cells. *Swiss Med Wkly* 2006; **136**: 552-556

4 **Menthena A**, Deb N, Oertel M, Grozdanov PN, Sandhu J, Shah S, Guha C, Shafritz DA, Dabeva MD. Bone marrow progenitors are not the source of expanding oval cells in injured liver. *Stem Cells* 2004; **22**: 1049-1061

5 **Howard BJ**, Pohorecki R, Becker GL, Landers DF. Energy status in anoxic rat hepatocytes: effects of isoflurane, solution composition, and hypothermia. *Liver Transpl Surg* 1995; **1**: 220-224

6 **Berry MN**, Halls HJ, Grivell MB. Techniques for pharmacological and toxicological studies with isolated hepatocyte suspensions. *Life Sci* 1992; **51**: 1-16

7 **Seglen PO**. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976; **13**: 29-83

8 **Tirnitz-Parker JE**, Tonkin JN, Knight B, Olynyk JK, Yeoh GC. Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. *Int J Biochem Cell Biol* 2007; **39**: 2226-2239

9 **Fausto N**, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev* 2003; **120**: 117-130

10 **He ZP**, Tan WQ, Tang YF, Zhang HJ, Feng MF. Activation, isolation, identification and in vitro proliferation of oval cells from adult rat livers. *Cell Prolif* 2004; **37**: 177-187

11 **Cantz T**, Manns MP, Ott M. Stem cells in liver regeneration and therapy. *Cell Tissue Res* 2008; **331**: 271-282

12 **Forbes SJ**. Stem cell therapy for chronic liver disease--choosing the right tools for the job. *Gut* 2008; **57**: 153-155

13 **Matsusaka S**, Tsujimura T, Toyosaka A, Nakasho K, Sugihara A, Okamoto E, Uematsu K, Terada N. Role of c-kit receptor tyrosine kinase in development of oval cells in the rat 2-acetylaminofluorene/partial hepatectomy model. *Hepatology* 1999; **29**: 670-676

14 **Yasui O**, Miura N, Terada K, Kawarada Y, Koyama K, Sugiyama T. Isolation of oval cells from Long-Evans Cinnamon rats and their transformation into hepatocytes in vivo in the rat liver. *Hepatology* 1997; **25**: 329-334

15 **Dumitriu IE**, Mohr W, Kolowos W, Kern P, Kalden JR, Herrmann M. 5,6-carboxyfluorescein diacetate succinimidyl ester-labeled apoptotic and necrotic as well as detergent-treated cells can be traced in composite cell samples. *Anal Biochem* 2001; **299**: 247-252

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BRIEF ARTICLES

Lung tissue flap repairs esophagus defection with an inner chitosan tube stent

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Author contributions: Chen G and Shi WJ contributed equally to this work; Shi WJ designed the research; Chen G and Shi WJ performed the research; Chen G contributed to the new chitosan tube stent; Chen G and Shi WJ analyzed the picture and wrote the paper.
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Abstract

AIM: To repair the partial esophagus defect with a chitosan stent, a new esophageal prosthesis made of pulmonary tissue with vascular pedicle.

METHODS: Fifteen Japanese big ear white rabbits were divided into experimental group (n = 10) and control group (n = 5). Esophagus defect in rabbits of experimental group was repaired using lung tissue flap with a chitosan tube stent, gross and histological appearance was observed at week 2, 4 and 8 after operation, and barium sulphate X-ray screen was performed at week 10 after operation. Esophagus defect of rabbits in control group was repaired using lung tissue flap with no chitosan tube stent, gross and histological appearance was observed at week 2, 4 and 8 after operation, and barium sulphate X-ray screen was performed at week 10 after operation.

RESULTS: In the experimental group, 6 rabbits survived for over two weeks, the lung tissue flap healed esophageal defection, and squamous metaplasia occurred on the surface of lung tissue flap. At week 10 after operation, barium sulphate examination found that barium was fluent through the esophagus with no stricture or back stream, the creeping was good. In the control group, 4 rabbits survived for two weeks, the lung tissue flap healed esophageal defection with fibrous tissue hyperplasia, barium sulphate examination found that barium was fluent through the esophagus with a slight stricture or back stream, and the creeping was not good at week 10 after operation.

CONCLUSION: Esophagus defect can be repaired using lung tissue flap with an inner chitosan tube stent.

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Key words: Lung; Tissue flap; Chitosan; Stent; Esophagus reconstruction

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INTRODUCTION

Esophagus disease is one of the common digestive tract diseases in our country. After excision, the esophagus needs to be reconstructed to restore the digestive tract continuity. At present, the commonly used esophagus reconstruction substitutes mainly include musculocutaneous flap with vessel peduncle[1], platysma musculocutaneous flap[2,3], periosteum intercostal muscle flap[4] and others[5-8] from stomach, colon and jejunum. Plastic tube, metallic pipe, teflon tube, silica gel tube, *etc*, are also used in reconstruction of artificial esophagus. However, their effects are not really ideal. The ideal substitute should be nontoxic, absorbable, without repulsive response and carcinogenicity, and easy to gain.

At present, various esophagus reconstruction techniques are available[9-11] and each has its own particular advantages and disadvantages. Irrespective of the kind of reconstruction, reducing scar formation and preventing stricture are still the key points. Different flaps from the lung tissue petal have been successfully applied in reconstruction of trachea[12]. Application of

lung tissue petal, inside lining metal and silica gel pipe stent in animals is also successful, but it is unable to overcome foreign matter response. In this study, we used inside lining metal, a new absorbable biological material-chitosan tube, to repair partial esophagus defect by preventing post-surgery stricture. Some questions concerning its application were discussed.

MATERIALS AND METHODS

Experimental animals and main materials

Fifteen healthy Japanese big ear white rabbits, weighing 3 kg, were provided by China Medical University Shengjing Hospital Animal Center. The rabbits were divided into experimental group (n = 10) and control group (n = 5). Chitosan tube stent, 15 mm long, 4 mm in inside diameter, was purchased from Shandong Province, China. Antiseptic glutaric dialdehydel were independently developed by the authors. TKR-200C micro-organism life-support machine was provided by Jiangxi Province, China. Rabbit surgery table, chest surgery instruments, infusion instruments, 20% urethane vein anesthetics, ketamine anaesthetics, 1% procaine, laryngoscope, 3.0-4.0 model trachea intubation were bought from Jiangxi Province, China. Tooth pad, medical adhesive plaster, digital camera (Sony, DSC-T10), and optical microscope (Olympics CH-20) were used in this study.

Experimental techniques

Before surgery, rabbits did not receive any medicine. Twenty percent of urethane vein anesthetics (5 mL/kg) was injected into abdominal cavity. Three minutes after anesthesia, the breath of rabbits was slow and changed to shallow and abdominal breath with corneal reflex. Ketamine (1 mg/kg) was intramuscularly injected to reduce the pain. The anaesthetized rabbits were fixed on the operation table at a supine position, and the assistant pulled in the flank with the bandage to draw in the front tooth and lower jaw. The mouth was pulled open with 1% procaine spraying. The throat was superficially anaesthetized. The operator stood in the rabbit head side, set the laryngoscope from one side of mouth, pushed away the tongue from tip to root. Epiglottis was exposed behind the tongue root resembling the white soft bone. Sometimes, vocal cord could not be found, but air bubbles could be observed. The trachea was gently pulled in intubation. The air current in the trachea pipe could be heard. When the inspiration was sent in gently, rapid vertical insertion was performed. Trachea intubation and tooth pad were fixed 13 cm away from the front teeth. When the air exhaled from the trachea intubation could be felt with hands, the life-support machine connected with oxygen was adjusted to a low current capacity.

Surgery method

The left side and barrier height of rabbit decubitus were exposed in the chest cavity, with the four limbs of rabbits fixed and the right flank prepared for operation of the chest. The first step was to cut open pleural membrane. When lung collapse was observed, the rabbits were given machinery ventilation, and the breath frequency was adjusted to 30 times/min, and then adjusted according to the lung inflation. A stomach tube was inserted into esophagus to support it, and the center-section was searched for its dissociation, and slung with a spun yarn cloth strip. The excision of central esophagus was an esophagus wall, 3 mm in diameter, to make a animal model of partial esophagus wall damage. In the experimental group, a chitosan tube was placed in breakage of the esophagus and fixed with a needle. The esophagus was wrapped by the nearby lung tissue petal to form an encystation in the breakage place, and the edge of esophagus breakage was sutured continuously with a 3-0 silk suture. Then, the stomach tube was withdrawn to release the stress and return the esophagus. The control group did not need any inside lining chitosan tube. No chest internal hemorrhage and lung air leakage were found. Transition to ventilation was performed several times before the last needle was inserted into the pleural membrane. At end of the inspiration, pleural cavity was closed. The rabbits received fluid diet and anti-inflammation treatment, fresh milk and normal diet a week after operation.

Observation of target

Observation of ordinary circumstances: Survival, feed, body weight, and complication were observed after operation.

Observation of body: If the experimental rabbits died in the observation period, prompt postmortem examination was performed to find the cause of death. The animals were killed at weeks 2, 4 and 8 after surgery, respectively. Scar formation and chitosan tube were observed.

Observation of histology: The surviving animals were executed at week 2, 4 and 8, respectively. The damaged patching lung tissue was stained with H&E. The growth of lung tissue petal was observed under optical microscope.

Barium meal: Ten weeks after operation, esophagus of survived rabbits was observed by barium meal to see whether the esophagus was unobstructed.

RESULTS

Animal survival

After operation, 6 animals in experimental group, and 4 animals in control group survived, respectively. Five animals and 1 animal died on the same day after operation, 1 animal died due to anesthesia 1 d after operation, 1 animal died of unhealed fistula on day 4 after operation, 1 animal died of infusion accident on day 7 after operation, and 1 animal died of malnutrition at week 2 after operation.

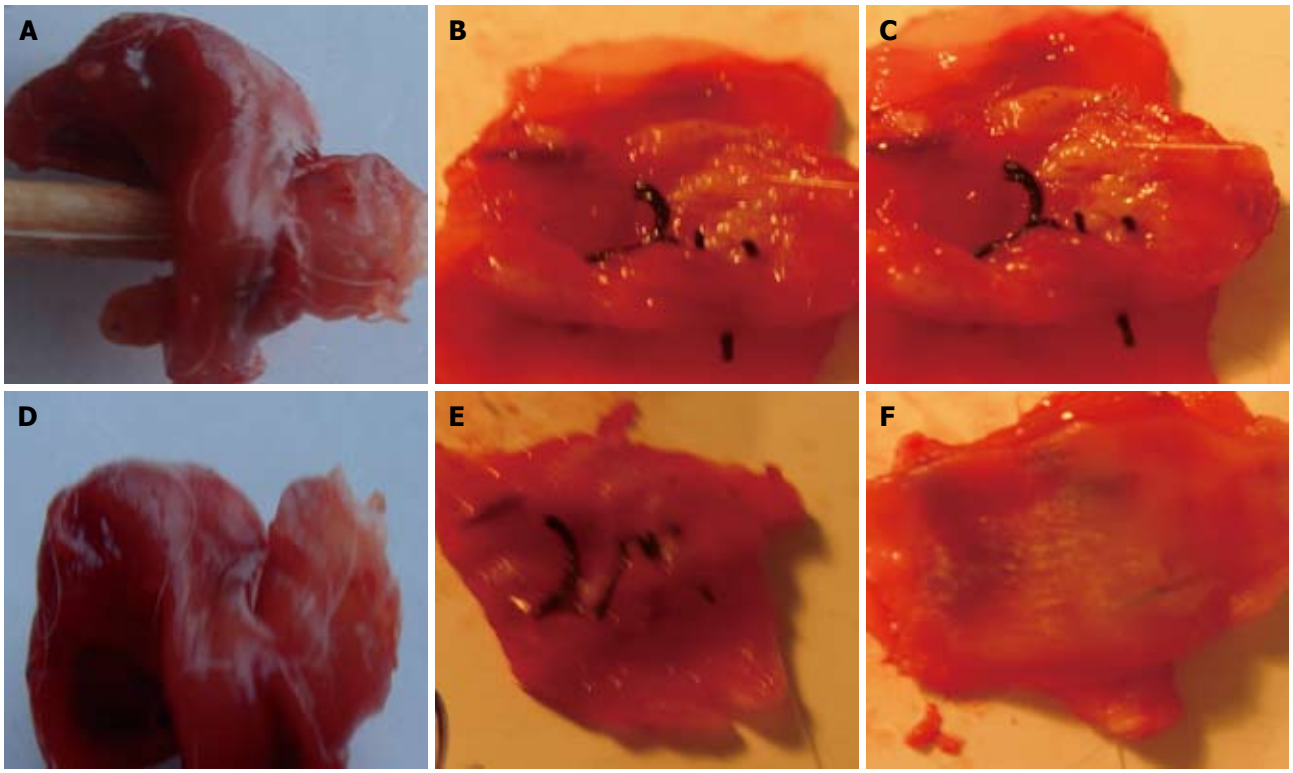

Figure 1 Lung tissue flap change in control group (A-C) and experiment group (D-F) after operation.

Observation of ordinary circumstances

Five days after operation, animals in experimental and control groups could have oral diet. The animals were provided with a small amount of food 5 d after operation and normal diet 10 d after operation. During this period, no obvious feed barrier was observed in animals of the experimental group. Three weeks after operation, animals in the control group had a poor appetite. One week after operation, the body weight of all animals decreased about 1-2 kg. The body weight of animals in the control group was still lighter than before operation.

Observation of body

Two weeks after operation, the esophagus damage was observed, the esophagus breakage edge and lung tissue petal were tightly united, accompanying dropsy. The chitosan tube could be touched under the substitute lung tissue petal and was soft, and the lumen surface had membranous contamination. Four weeks after operation, the damage was completely repaired, the suture was not absorbed. In the experimental group, the internal lumen surface of esophagus substitute was smooth, the blood circulation was rich, and the chitosan tube was partly decomposed. In the control group, the damage was red in color, hyperplasia was found around the damage, accompanying dropsy. In the experimental group, eight weeks after operation, the esophagus defect was covered by the white thick membranous substance. No dropsy, necrosis and ulcer, obvious stricture, or chitosan cast were found in the pipe wall. In the control group, scar formation and hyperplasia were found on tissue petal surface (Figure 1).

Two weeks after operation, the lung structure of rabbits in experimental and control groups was observed. Pulmonary alveoli were withered and collapsed, and the denatured pulmonary alveolus cells were tumescent with acute inflammation response. Four weeks after operation, atypical pulmonary alveolus structure was observed in the experimental group. The inflammatory response was weakened accompanying a few neutral granular cells and lymphocytes, but no obvious fiber proliferation was observed. In the control group, the central pulmonary alveolus structure was atypical accompanying fiber cells and a few inflammatory cells. Eight weeks after operation, a large number of squamous epidermis cells were observed on the surface of lung tissue petal, and the chronic inflammatory response was significantly decreased in the experimental group. The control group had chronic inflammation response, accompanying obvious fiber proliferation but no superficial squamous metaplasia (Figure 2).

Barium meal

Ten weeks after operation, esophagus barium test was performed. In the experimental group, barium meal went smoothly through the esophagus. No obvious stricture, reverse flow, anastomotic stoma leakage and expansion were observed in the esophagus. The creeping motion was good. Mild stricture was observed in control group. The anastomotic stoma was healed with expansion, and the barium meal went through smoothly with general creeping motion (Figure 3).

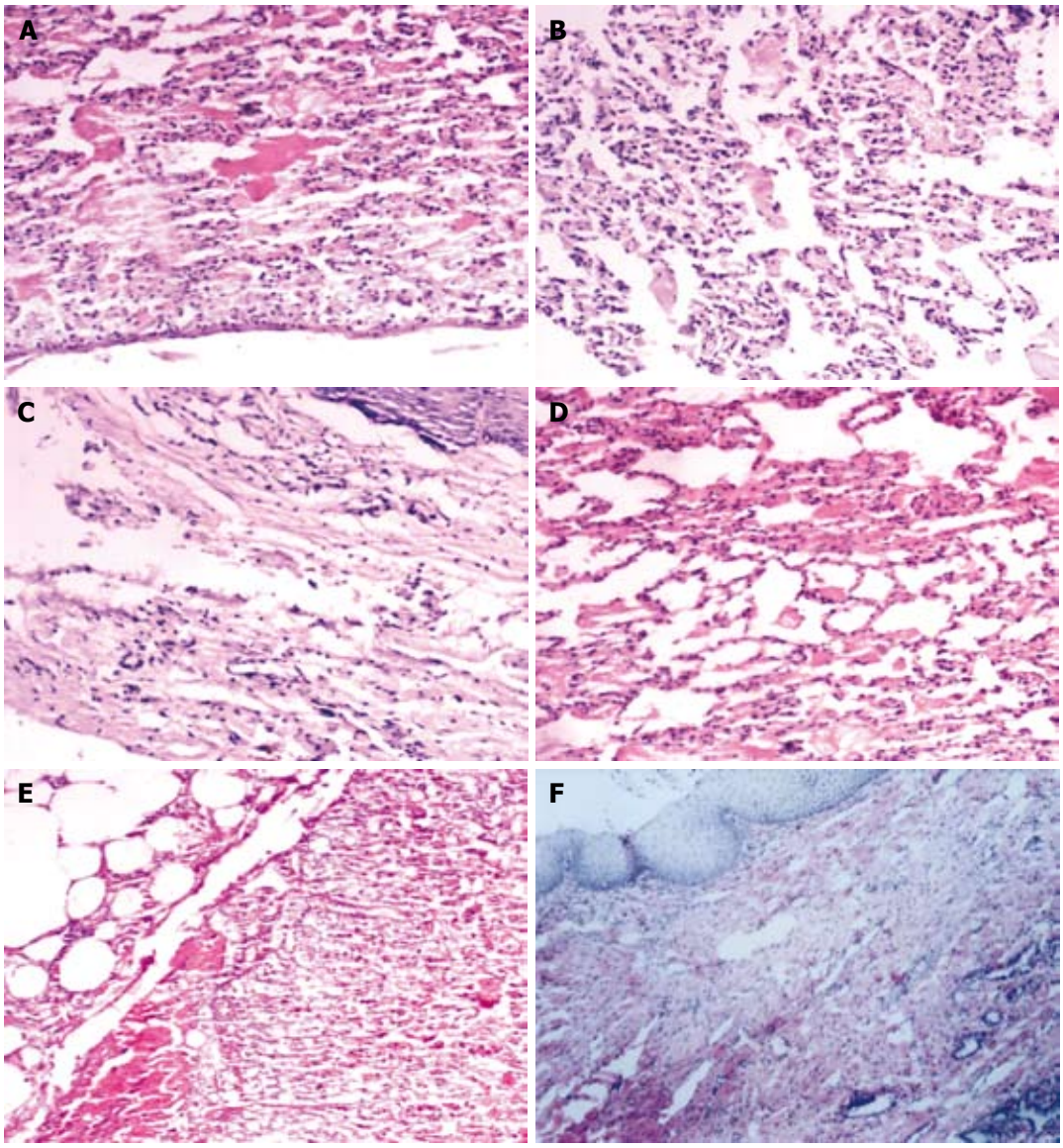

Figure 2 Lung tissue flap change under optical microscope in control group (A-C) and experimental group (D-F) after operation (HE, × 10).

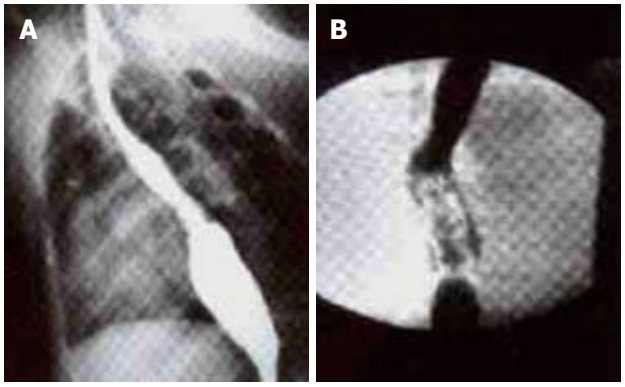

Figure 3 Results of esophagus barium test in control group (A) and experimental group (B) ten weeks after operation.

DISCUSSION

Esophagus excision and reconstruction are important in esophagus surgery. In recent years, biological substitutes have been extensively studied[13-21]. Zhi *et al*[13] reported that biological artificial esophagus can be used to repair esophagus defect. In their study, esophagus substitution test was performed in 30 experimental dogs, showing that esophagus substitution can repair 93.3% of esophagus defects. Zhang *et al*[14-17] excised chest esophagus at the right chest flank of 30 Chinese hybrid dogs, and an 8 cm long biological artificial esophagus was used to reconstruct esophagus. At present, we still face the problems of fistula, stricture, and length of implants, which need further research. Based on the biological esophagus substitute, biodegradable and non-biodegradable materials have been developed for making artificial esophagus[18,19]. Meanwhile, the degradation speed of biodegradable material matches that of non-degradation material, which exerts the supporting function and prolongs the supporting time of biological materials, and finally regenerates esophagus to completely substitute the artificial esophagus. The project of digestion tube has made certain progress. However, its effect is unknown. Further study is needed on implantation and production of epithelial and muscle cells, gland and nerve plexus regeneration, scar reduction, speeding up repair and regeneration of

structure, *etc*[20].

Dr. Shi first used lung tissue petal substitution to repair trachea defect successfully[21], which is a new direction to repair esophagus defect with lung tissue petal and reconstruct esophagus. In the earlier experiment, we wrapped the damage spot using the lung tissue petal to make the artificial esophagus, in order to prevent stricture after repair of esophagus defect[22,23]. Based on previous experiment results, we chose a chitosan material to make absorbable tube stent to overcome the rejection, and further explored the feasibility of this new method. The blood circulation of lung tissue petal was good, which was confirmed by pathology and electron microscopy. The compatibility of repair material was good, thus avoiding foreign matter rejection and forming reliable scars of fibers. The lung tissue petal has certain ductility, and different lobes of the lungs can be selected to make different lengths of lung tissue petal. In brief, the selection of lung tissue petal is convenient, the compatibility is good and the blood circulation is rich, with a high scar formation ability and good anti-infectiousness, and good environment for esophagus epidermis.

Chitin is the only high polymer material with widespread biodegradation[24]. Acetyl-escaped product, also known as chitosan[25], has the good biological compatibility with animal organs and cells, and degrades the low molecular oligosaccharide with no accumulation of products *in vivo* and no immunogenic ability. The tube we made of it is elastic and tough, slightly soft when it meets water, and can suppress inflammation response, prevent adhesion and scar formation in the breakage site. It was reported that chitosan membrane can prevent the adhesion to peritoneum and thus can be used in clinical practice[26]. It was also reported that chitosan can insert into nerve tube stent[27,28]. Chitosan tube stent is degraded gradually and absorbed *in vivo*, with no toxicity, stimulation and rejection. It has been shown that epidermis of the esophagus has certain degree of stricture but it is not serious enough to cause feed barrier in esophagus of dogs[29]. Long-term survival and delayed chronic inflammation have been achieved using metallic pipe and silica gel tube as an inner lining support, but foreign matter rejection occurs. We used chitosan as a support to prevent scar formation and stricture of esophagus by making use of its compatibility and degradability. When stricture is formed, chitosan tube is degraded *in vivo*, and its product is not toxic and has no side effects. Thus, it is worthy to be further studied. The most serious complication is fistula, which occurs 5-7 d after operation and is related with infection, anastomotic techniques and blood circulation, *etc*. Infection is the most important factor for the occurrence of complication, because esophagus patching is a pollutant. Since we performed the surgery under strict asepsis, we solved the problem. After operation, the rabbits in experimental group were fasted for 5 d, and then received venous transfusion and anti-inflammation treatment. Because the resistance of rabbits was lower than that of dogs, the mortality rate of rabbits was 30%. Since this study was to verify the new method and explore experimental conditions, a large sample size of slightly bigger animals, like dogs, pigs, *etc*, should be used in the study. We used big white rabbits to establish esophagus partial damage model, and repaired esophagus wall partial damage using chitosan tube with lung tissue petal as its inside lining. Based on results of this experiment, pigs and dogs and other bigger animals, may be further tested for the replacement of chitosan tube in the entire esophagus. Our experiment did not operate chest of rabbits. Compared with big animals, rabbits are docile, convenient, inexpensive, and easy to obtain. The choice of support is the key to the maintenance of unobstructed lumen, protection of the surface granulation tissue of lung from adhesion, formation of diverticulum and false passage. The metal lattice support is widely applied in treatment of esophagus stenosis, mainly because of its good support effect. Therefore, we used the chitosan tubular support, which functions as a support, reduces inflammation response, and can be absorbed and degraded by organisms, and can be sued as a substitute of esophagus.

Further study involving smooth muscle, nerve plexus and gland regeneration is needed[30]. Creeping motion restoration, long esophagus reconstruction, *etc*, can be achieved in clinical practice.

COMMENTS

Background

At present, esophagus reconstruction techniques are available, but each of them has its advantages and disadvantages. Reducing scar formation and stricture is still the key to esophagus reconstruction.

Research frontiers

In this study, chitosan tube was used as a support to prevent post-operation stricture.

Innovations and breakthroughs

Trachea was reconstructed using chitosan and silica gel pipe stent. A chitosan material was used to make absorbable support tubes to overcome the rejection and prevent stricture of esophagus.

Applications

Chitosan tubes provide a new surgery method for patients in whom substituting esophagus with cavity internal organs is impossible.

Terminology

Lung tissue petal: A lung lobe closing the segment of trachea.

Peer review

It is an interesting animal experiment about the use of chitosan stent with a lung tissue flap to repair esophageal perforation.

REFERENCES

1 **Chen HC**, Kuo YR, Hwang TL, Chen HH, Chang CH, Tang YB. Microvascular prefabricated free skin flaps for esophageal reconstruction in difficult patients. *Ann Thorac Surg* 1999; **67**: 911-916

2 **Wang RW**, Jiang YG, Gong TQ, Zhou JH, Zhao YP, Ma Z. Reconstruction of cervical esophageal stenosis with platysma myocutaneous flap. *Disan Junyi Daxue Xuebao* 2007; **29**: 749-751

3 **Zhao YP**, Wang RW, Jiang YG, Gong TQ, Zhou JH, Tan QY. Postoperative complication of reconstruction or repair defect of cervical esophagus using platysma myocutaneous flap. *Zhongguo Xiongxinxueguan Waike Linchuang Zazhi* 2001; **8**: 169-171

4 **Zhang X**, Wang XR, Li RH, Hu JG, Hou JS, Zhou SJ. Osteoperio-inercostal muscle pedicle flap: an effective material in esophagoplasty. *Zhonghua Xiongxinxueguan Waike Zazhi* 2000; 16: 39-40
5 **Uehara M**, Helman JI, Lillie JH, Brooks SL. Blood supply to the platysma muscle flap: an anatomic study with clinical correlation. *J Oral Maxillofac Surg* 2001; **59**: 642-646
6 **Ariyan S**. The transverse platysma myocutaneous flap for head and neck reconstruction: an update. *Plast Reconstr Surg* 2003; **111**: 378-380
7 **Zhou JH**, Jiang YG, Wang RW, Lin YD, Gong TQ, Zhao YP, Ma Z, Tan QY. Management of corrosive esophageal burns in 149 cases. *J Thorac Cardiovasc Surg* 2005; **130**: 449-455
8 **Jiang YG**, Lin YD, Wang RW, Zhou JH, Gong TQ, Ma Z, Zhao YP, Tan QY. Pharyngocolonic anastomosis for esophageal reconstruction in corrosive esophageal stricture. *Ann Thorac Surg* 2005; **79**: 1890-1894
9 **Schettini ST**, Pinus J. Gastric-tube esophagoplasty in children. *Pediatr Surg Int* 1998; **14**: 144-150
10 **ul-Haq A**, Tareen F, Bader I, Burki T, Khan NU. Oesophageal replacement in children with indolent stricture of the oesophagus. *Asian J Surg* 2006; **29**: 17-21
11 **Liao B**. Clinical analysis of esophageal stricture in child. *Zhongguo Xiongxinxueguan Waike Linchuang Zazhi* 2008; **15**: 71-72
12 **Shi WJ**, Zhang SN, Yang W, Zhao JG, Zhao Y, Liu J. [Clinical application and animal experiment of thoracic tracheal reconstruction by using pulmonary tissue flap] *Zhonghua Waike Zazhi* 2003; **41**: 218-221
13 **Zhi FC**, Zhang LJ, Peng XF, Wu XH, Pan DS, Wan TM, Liu SD, Zhang ZS, Zhou DY. Experimental study of esophagus reconstruction with biological artificial esophagus in dogs. *Zhonghua Xiaohua Zazhi* 2003; **23**: 3-7
14 **Zhang LJ**, Zhi FC, Rong TH, Peng XF, Wen DD, Yan SQ. Experimental study of the biological artificial esophagus in dogs. *Zhonghua Weichang Waike Zazhi* 2001; **4**: 157-160
15 **Zhang LJ**, Rong TH, Su XD, Lin P, Long H, FU JH. Experimental replacement of thoracic esophageal segment with a biomaterial artificial esophagus in dogs. *J Med Coll PLA* 2008; **23**: 1-8
16 **Zhang LJ**, Rong TH, Wu QL, Su XD, Long H, Zhao JM, Zhang PY, Li XD. [Histological regeneration process of "neo-esophagus"] *Ai Zheng* 2006; **25**: 689-695
17 **Zhang LJ**, Rong TH, Xu GL, Su XD, Zhi FC, Guo XM, Zhang PY. [Experimental study of preventing postoperative stenosis by modifying artificial esophagus in dogs] *Zhongguo Yixue Kexueyuan Xuebao* 2006; **28**: 325-328
18 **Yang LZ**, Hong ZP. Progress of the Research of Artificial Esophagus. *Zhonghua Xiongxinxueguan Waike Zazhi* 2006; **13**: 188-191
19 **Watanabe M**, Sekine K, Hori Y, Shiraishi Y, Maeda T, Honma D, Miyata G, Saijo Y, Yambe T. Artificial esophagus with peristaltic movement. *ASAIO J* 2005; **51**: 158-161
20 **Hori Y**, Nakamura T, Kimura D, Kaino K, Kurokawa Y, Satomi S, Shimizu Y. Effect of basic fibroblast growth factor on vascularization in esophagus tissue engineering. *Int J Artif Organs* 2003; **26**: 241-244
21 **Chen G**, Shi WJ. Clinical application of thoracic tracheal reconstruction by using pulmonary tissue flap combined with nickel-titanium alloy stent. *Shandong Yiyao* 2008; **48**: 12-13
22 **Zhao JG**, Shi WJ, Zhang SN, Han Y, Zhao Y, Liu J. Replacement of partial esophageal defect with pulmonary tissue with vascular pedicle. *Zhonghua Xiongxinxueguan Waike Zazhi* 2003; **19**: 166-168
23 **Yang W**, Shi WJ, Zhang SN, Han Y. Research of pulmonary flap substitutes for the esophagus in dogs. *Zhongguo Yike Daxue Xuebao* 2002; **31**: 7-9
24 **Berglund JD**, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials* 2003; **24**: 1241-1254
25 **Freier T**, Montenegro R, Shan Koh H, Shoichet MS. Chitin-based tubes for tissue engineering in the nervous system. *Biomaterials* 2005; **26**: 4624-4632
26 **Ao Q**, Wang AJ, Sun ZG, Zhang XF. Preparation and characterization of a chitosan conduit for neural tissue engineering. *Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu* 2008; **12**: 47-50
27 **Holland TA**, Tabata Y, Mikos AG. Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering. *J Control Release* 2005; **101**: 111-125
28 **Zhang WF**, Chen XG, Li PW, He QZ, Zhou HY. Preparation and characterization of theophylline loaded chitosan/beta-cyclodextrin microspheres. *J Mater Sci Mater Med* 2008; **19**: 305-310
29 **Liu J**, Shi WJ, Zhang SN, Han Y, Zhao JG. Experimental research of esophagus replacement with pulmonary flap in dogs. *Zhongguo Xiufu Chongjian Waike Zazhi* 2006; **20**: 507-510
30 **Qin X**, Xu ZF, Zhao XW, Shi HC, Zhou JH, Sun K, Gao XY. Reconstruction of a cervical esophagus segment with an artificial prosthesis by use of a polyurethane stent covered with collagen-chitosan sponge in dogs. *Zhongguo Xiufu Chongjian Waike Zazhi* 2003; **17**: 374-377

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BRIEF ARTICLES

Biotransformation of aesculin by human gut bacteria and identification of its metabolites in rat urine

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Abstract

AIM: To observe the biotransformation process of a Chinese compound, aesculin, by human gut bacteria, and to identify its metabolites in rat urine.

METHODS: Representative human gut bacteria were collected from 20 healthy volunteers, and then utilized *in vitro* to biotransform aesculin under anaerobic conditions. At 0, 2, 4, 8, 12, 16, 24, 48 and 72 h post-incubation, 10 mL of culture medium was collected. Metabolites of aesculin were extracted 3 × from rat urine with methanol and analyzed by HPLC. For *in vivo* metabolite analysis, aesculetin (100 mg/kg) was administered to rats *via* stomach gavage, rat urine was collected from 6 to 48 h post-administration, and metabolite analysis was performed by LC/ESI-MS and MS/MS in the positive and negative modes.

RESULTS: Human gut bacteria could completely convert aesculin into aesculetin *in vitro*. The biotransformation process occurred from 8 to 24 h post-incubation, with its highest activity was seen from 8 to 12 h. The *in vitro* process was much slower than the *in vivo* process. In contrast to the *in vitro* model, six aesculetin metabolites were identified in rat urine, including 6-hydroxy-7-gluco-coumarin (M1), 6-hydroxy-7-sulf-coumarin (M2), 6, 7-di-gluco-coumarin (M3), 6-glc-7-gluco-coumarin (M4), 6-O-methyl-7-gluco-coumarin (M5) and 6-O-methyl-7-sulf-coumarin (M6). Of which, M2 and M6 were novel metabolites.

CONCLUSION: Aesculin can be transferred into aesculetin by human gut bacteria and is further modified by the host *in vivo*. The diverse metabolites of aesculin may explain its pleiotropic pharmaceutical effects.

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Key words: Aesculin; Biotransformation; Human gut bacteria; Rat urine; Sulfated derivatives; LC/ESI-MS; Aseculetin

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INTRODUCTION

Aesculin, a 6, 7-dihydroxy derivative of coumarin with pleiotropic pharmacological and biochemical properties, has recently been analyzed for its biochemical activity[1]. Kaneko and colleagues[2] found that aesculetin and its 6-glycoside, aesculin, can inhibit oxidative DNA damage and formation of aberrant crypt foci and tumors. Furthermore, this compound shows an inhibitory effect on BOP-induced oxidative DNA damage and carcinogenesis in a hamster pancreatic tumor model[2], as well as chemo-preventive[3] and anti-tumor activity on cancer[4]. Aesculin and aesculetin have strong antioxidative and photo-protective activities, by scavenging 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and superoxide anions from the xanthine/xanthine oxidase system and inhibiting oxidation of 5-(6-)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate[5]. Aesculetin increases apoptosis of 3T3-L1 adipocytes in a time- and

dose-dependent manner[6], and inhibits cell growth and cell cycle progression by inducing G1 phase arrest in HL-60 leukaemia cells, which is a direct result of the inhibition of retinoblastoma protein phosphorylation[7]. Aesculetin also displays multiple immunomodulatory effects on murine lymphocytes and peritoneal macrophages in rat liver[8], including anti-inflammatory activity, inhibition of lipoxygenase and tyrosinase activity, scavenge of hydroxyl radicals and suppressing lipid peroxidation.

However, the biotransformation progress of aesculin and its derivatives has not been completely determined, let alone their complex structure-activity relationships. Aesculetin is one of the simplest coumarins with two hydroxyl groups at carbons 6 and 7 that serve as targets for O-methylation or O-glycosylatin. It has been reported that biotransformation of aesculetin with *E. coli* expressing O-methyltransferase (POMT-9) generates scopoletin, isoscopoletin, and scoparone[9]. The growth of *E. coli* is inhibited by aesculetin, but not by aesculin[10]. Hairy roots of medicinal morning glory (Pharbitis nil) show a potent glucosylation activity against aesculetin, especially at 7-hydroxyl group[11]. As one of the metabolites of caffeic acid oxidation, aesculetin observed in perfused rat liver may be responsible for its biological effects observed *in vivo*[12]. Aesculetin is a substrate of mushroom polyphenol oxidase and horseradish peroxidase enzyme resulting in the oxidization of aesculetin into its o-quinone[13]. Despite these accumulated data, we still do not fully understand the structural basis and dynamic pattern underlying the pleiotropic biological effects of aesculetin and there are few reports describing its derivatives or metabolites.

Therefore, in the present work, we analyzed the biotransformation process of aesculin in response to human gut microflora in a rat model, in order to identify its novel metabolites, which may enrich our understanding of the complicated structure-activity relationships between aesculin and its metabolites.

MATERIALS AND METHODS

Collection of representative human gut bacteria

Twenty healthy volunteers (10 males and 10 females) at the age of 20-24 years, were recruited from students of Chengdu University of Traditional Chinese Medicine. Their faeces were collected and intestinal flora was identified as normal with an automatic system of bacteria identification (BD, USA). Then, fecal samples were pooled. The bacterial concentration was adjusted to 2×10^7 CFU/mL in culture medium, and 1 mL of aliquots was stored at -80℃ until use.

Preparation of anaerobic culture medium

Culture medium for anaerobic gut bacteria, GAM, was prepared according to the following formula: 3 g of soybean phytone, 10 g of peptone, 13.5 g digested blood serum powder, 5 g yeast extract, 2.2 g extractum carnis, 1.2 g beef liver extract, 3 g glucose, 5 g soluble starch, 2.5 g KH_2PO_4, 3 g NaCl, 0.3 g L-cysteine, 0.3 g sodiumthioglycollate, were added into 800 mL of dH_2O. The pH was adjusted to 7.4 with 1 mol/L NaOH and the final volume was adjusted to 1000 mL with dH_2O. The medium was sterilized by autoclaving at 121℃ for 20 min and stored at 4℃ for further use.

Chemicals

Aesculin (6-hydroxy, 7-glycoside coumarin), with an estimated purity of 98%, was purchased from Aldrich Chemical Co.(Milwaukee, WI). For the preparation of standard aesculin, 250 mg of aesculin was added to 25 mL of saline (0.9% NaCl solution) and dispensed into the solution by sonication. The mixture was then boiled for 10 min to dissolve aesculin. This stock solution of aesculin (10 mg/mL) was stored in the dark at -20℃ until use.

Aesculetin (6, 7-dihydroxycoumarin) was also purchased from Aldrich Chemical Co. (Milwaukee, WI). The stock solution of aesculetin (10 mg/mL) was prepared as above and stored under the same condition for aesculin.

Exposure of aesculin/aesculetin to human gut bacteria

Twenty millilitre of aseculin stock solution was added to 179 mL of GAM in a 500 mL glass flask, followed by addition of 1 mL of human gut bacteria. The final concentration of aesculin was 1 mg/mL. The mixture was incubated at 37℃ in DY-II anaerobic incubator containing 80% N_2, 10% CO_2, and 10% H_2 (Yiwu Co. Ltd, Zhejiang Province, China). Ten mL of the solution was removed at 0, 2, 4, 8, 12, 16, 24, 48 and 72 h post-incubation, respectively, and 10 mL of methanol was then added to at each time point. The solutions were mixed well with hands for 5 min and the upper phase was transferred into a fresh flask. This extraction process was repeated two times and all the upper phase fractions were pooled for HPLC analysis.

The same protocol was employed to incubate aesculetin with human gut bacteria.

HPLC analysis of cultured aesculin/aesculetin

Preparation of control stock solution: Methanol was used as a solvent to prepare the aesculin and aesculetin stock solutions. The final concentration of aesculin and aesculetin was 10.08 mg/mL and 6.120 mg/mL, respectively. The solutions were kept in the dark at 4℃ for up to one week.

HPLC was performed on a Waters Separation Module 2695 at a detection wavelength of 340 nm, at a screen wavelength of 210-400 nm, at a flow rate of 1.0 mL/min, at a sample size of 10 μL, and at a column temperature of 25℃, with mobile phase A set at 5% acetic acid : methanol (78:22), mobile phase B at 5% acetic acid: methanol gradient elution (0-20 min, 80:20; 20-35 min, linearity changed from 80:20 to 40:60), and mobile phase C at 5% acetic acid: methanol gradient elution (0-20 min, 90:10; 20-35 min, linearity changed from 90:10 to 50:50), respectively.

Preparation of urinary metabolites

Twelve adult SD rats (6 males and 6 females), weighing 200 ± 5.6 g, were raised under specific pathogen-free conditions at the University Laboratory Animal Service

Table 1 Quantitative biotransformation effeciency of human gut bacteria for aesculin

Incubation time (h)	0	2	4	8	12	16	24	48	72
Aesculin Conc. (mg/mL)	0.886	0.848	0.832	0.873	0.166	0.043	ND	ND	ND
Aesculetin Conc. (mg/mL)	ND	ND	ND	ND	0.332	0.418	0.434	0.466	0.462
Transformation ratio (%)	0	0	0	0	79.20	94.90	100	100	100

"ND" indicates that nothing was tested.

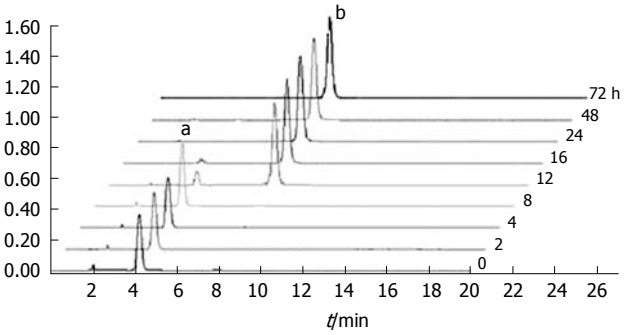

Figure 1 Biotransformation of aseculin by anaerobic human gut bacteria. The gut bacteria comprised 20 pooled fecal samples from healthy volunteers. Incubation was performed under anaerobic conditions (80% N2, 10% CO_2, 10% H2) at 37℃ for defined time periods. Ten millilitre of samples was collected at 0, 2, 4, 8, 12, 16, 24, 48 and 72 h, respectively post-incubation. Aesculin and metabolites were then extracted with methanol and analyzed by HPLC. The vertical axis shows the relative values for the concentration of aesculin and/or its metabolite. The horizontal axis indicates the HPLC overflow time.

Center of Chengdu University of Traditional Chinese Medicine, China. Each rat was raised in a solitary metabolic cage, with free access to normal food in a 12 h light and dark cycle. After adaptive nurture for one week, the aesculetin solution was administrated at a dosage of 100 mg per kilogram. Rat urine produced from 6 to 48 h after administration of aesculetin solution was collected for analysis. Five millilitre of urine sample from each rat was mixed for HPLC analysis.

Identification of aesculin metabolites in rat urine by LC/MS

Aesculin metabolites were identified in rat urine by XenoBiotic Laboratories, Inc., Plainsboro, NJ, USA. Control solutions of aesculetin and aesculin, and rat urine sample were analyzed by LC/ESI-MS and MS/MS in the positive and negative modes. The actual instrument conditions were modified to optimize chromatography and instrument sensitivity.

HPLC: HPLC System:Waters Separation Module 2695. Column: Ace 3, C18, 3 μm, 150 mm × 4.6 mm; guard column: Ace 3, C18, 10 × 3.2 mm; column temperature: 25℃; autosampler temperature: 4℃; mobile phase A: 0.4% HCOOH in H_2O; mobile phase B: CAN.

MS: Mass spectrometer: Finnigan LCQ™ mass spectrometer. Data system: ThermoQuest Xcalibur Version1.3; ionization mode: positive or negative electrospray ion modes [(+)/(-) ESI]; ion spray (IS): 4.5 kV; capillary temperature: 240℃; sheath gas flow: N_2, ~80 units; auxiliary gas flow: N_2, ~20 units; collision gas: helium.

RESULTS

Human gut bacteria converted aesculin into aesculetin

HPLC analysis revealed that our representative human gut bacteria degraded the glycoside of aesculin, thus completely converting it into aesculetin (Figure 1). The biotransformation process occurred between 8 and 24 h post-culture. After 24 h, aesculin was almost transferred into aesculetin. No other metabolite was observed, indicating that human gut bacteria cannot further modify aesculetin. No conversion of aesculin to aesculetin was observed in the absence of human gut bacteria 72 h after incubation (data not shown), indicating that human gut bacteria are a prerequisite for the biotransformation of aesculin.

The biotansformation efficiency of aesculin was quantitatively determined according to the following equation: Transformation ratio= (aesculetin concentration/178)/(aesculetin concentration/178 + aesculin concentration/340), where 178 represents the molecular weight of aesculetin and 340 represents the molecular weight of aesculin. Almost 80% of aesculin biotransformation occurred from 8 to 12 h post-incubation (Table 1).

The mixture of human gut bacteria did modify aesculetin under the same culture conditions (data not shown), indicating that human gut bacteria can only biotransform aesculin but not further modify its molecules.

Identification of aesculetin metabolites in rat urine

Six metabolites of aesculin were detected in rat urine (Figure 2). The primary characteristics of each metabolite were obtained by CAD-MS/MS analysis (Figure 3). One or two hydrogens in these metabolites [6-hydroxy-7-gluco-coumarin (M1), 6-hydroxy-7-sulf-coumarin (M2), 6, 7-di-gluco-coumarin (M3), 6-glc-7-gluco-coumarin (M4), 6-O-methyl-7-gluco-coumarin (M5) and 6-O-methyl-7-sulf-coumarin (M6)] of the hydroxyl groups of aesculetin were modified by glucosylation, glycoside, sulfation, and/or methylation. In the present study, M2 and M6 were found to be novel aesculin metabolites compared with the reported data[1,2].

Compared with the reference standards for aesculetin and aesculin, six aesculetin metabolites in rat urine were clearly observed on HPLC UV-chromatogram (Figure 4). The spectral data of aesculetin using LC/(+)ESI-MS and

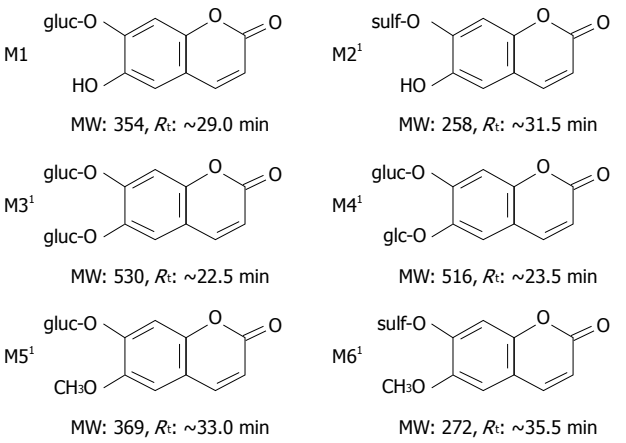

Figure 2 Summary of aesculetin metabolites identified in rat urine. [1]The position(s) of methylation and/or conjugation(s) may be exchangeable on the two phenols.

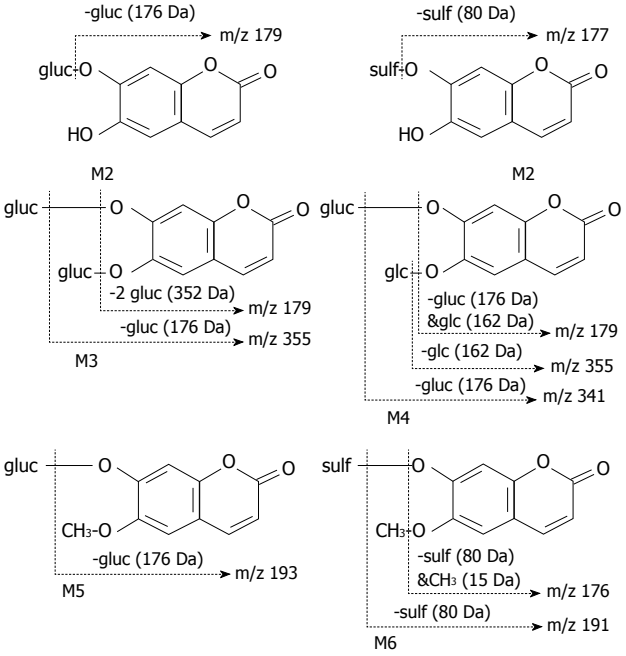

Figure 3 Proposed characteristic (-) CAD-MS/MS fragmentations for aesculetin metabolites.

MS/MS are shown in Figure 5. By comparing the data, we could identify the substitution position of aesculetin metabolites in rat urine *in vivo*.

Unlike gut microflora, mammalian physiology could further modify aesculin and the pharmaceutical effects of aesculin might be a result of these metabolic modifications.

DISCUSSION

Natural coumarins, widely distributed in plants, fungi and bacteria, have pleiotropic biological effects. Coumarins, made of fused benzene and a-pyrone rings, have phenolic hydroxyl groups in their structures that can be easily replaced and/or modified, forming various metabolites or derivatives. Aesculin, one of the simplest

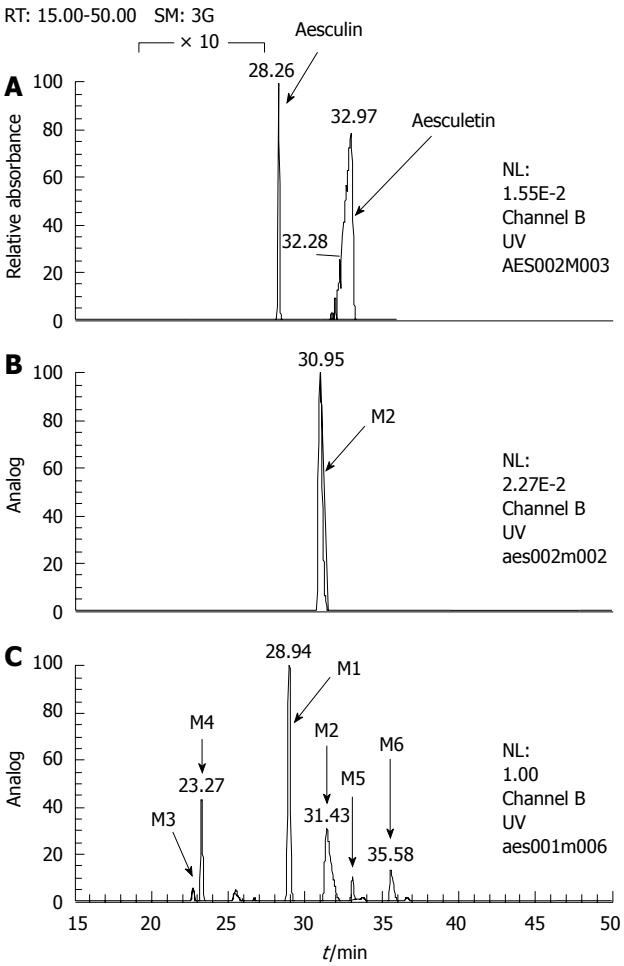

Figure 4 HPLC UV-chromatograms of reference standards for aesculetin and aesculin (A), M2 isolated from rat urine (B), and a rat urine sample dosed with aesculetin with assignments of aesculetin urinary metabolites (C).

coumarins known to have multiple pharmacological and biochemical activities, is an established inhibitor of lipoxygenase and cyclo-oxygenase and shows scavenging effects on ROS[13]. It has been reported that aesculetin has chemo-preventive and anti-tumor activities i*n vivo* against cancer[5,14] and also induces apoptosis in several types of human cancer cells by diverse pathways[1,6,7,15,16]. Hence, it is a great challenge to determine the complex structure-activity relationships between aesculin/aesculetin and their metabolites. The aesculin metabolites in rat urine were identified both *in vitro* and *in vivo*.

The results of this study show that human gut bacteria could completely biotransform aesculin into aesculetin. About 80 % of aesculin was converted into aesculetin in less than 12 h post-incubation. The reason why the *in vitro* biotranformation speed of aesculin was so slow remains unclear. The universal time lag of our bacterial culture conditions might be a major reason, since human gut bacteria must first synthesize enough enzymes in order to adapt to any new environment. Since the starting concentration (10^5 CFU/mL) of human gut bacteria was markedly lower than that of human gut flora (about 10^{12} CFU/gram), some additional time is necessary for the exponent growth of seeded bacteria in order to reach the

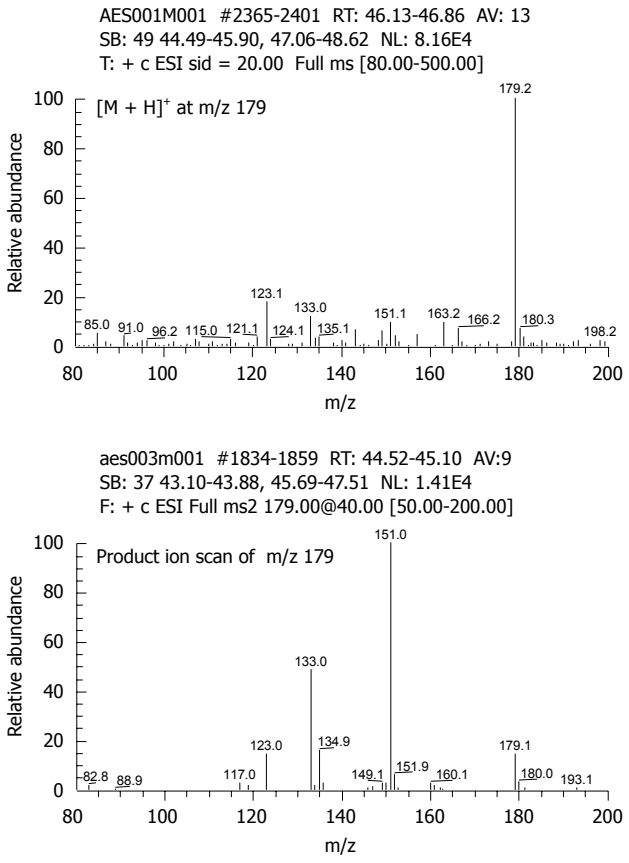

Figure 5 Aesculetin LC/(+)ESI-MS and MS/MS spectral data.

maximum bacterial concentration in the culture medium. In addition, we only employed anaerobic gut bacteria for the biotransformation of aesculin, which should be somewhat slower than *in vivo* conditions, since both anaerobic and aerobic bacteria may be involved in the *in vivo* aesculin biotransformation process.

The results of aesculin transformation suggest a simple but vivid example of host-gut bacteria cooperation in the dynamic utilization and subsequent modification of xenobiotics. Human gut bacteria degrade the glycoside of aesculin to facilitate its absorbtion in intestine, then modification of aesculetin into various derivatives with the potential of having multiple biological activities, occurs in the host. It was reported that the distal human intestine represents an anaerobic bioreactor programmed with a large population of bacteria, providing us with genetic and metabolic attributes, including the ability to harvest otherwise inaccessible nutrients and to metabolize diverse xenobiotics[17-19]. Microbiome is still a largely under explored regulator of drug metabolism and bioavailability[17-19]. Our data present here provide additional evidence for the metabolism of aesculin by human gut microbiota.

In this study, two novel sulfated aesculetin metabolites were identified in rat urine, which might represent very interesting modifications of coumarins. As aesculetin has two hydroxyl groups at carbons 6 and 7, it can serve as targets for O-methylation or O-glycosylatin. It has been reported that O-methylated products of aesculetin are scopoletin (6-O-methyl aesculetin), isoscopoletin (7-O-methyl aesculetin), and scoparone (6, 7-O-dimethyl aesculetin), which possess antimicrobial, immunosuppressive, and hypolipidermic activities[7,9]. The hydroxyl groups of aesculetin, especially the 7-hydroxyl group, can also be glucosylated in plants such as hairy roots of medicinal morning glory[11]. In addition, mushroom polyphenol oxidase (PPO) and horseradish peroxidase (POD) can oxidize aesculetin and generate its o-quinone[13]. However, as far as we are know, no sulfated derivative of aesculetin has been identified. Indeed, sulfation is one of the most important modifications in many natural compounds. As their sulfate groups accumulate negative charges, sulfated cumarins can interact with certain molecular domains that usually have positive charges, resulting in compounds with anti-viral, anti-tumor and anti-oxidative effects[12]. Therefore, these novel derivatives identified in rat urine offer new evidence for the biological activities ascribed to aesculetin.

COMMENTS

Background

Aesculin and its metabolites have pleiotropic pharmacological and biochemical properties, such as antioxidative, photo-protective and multiple immunomodulatory effects. However, their biotransformation has not been extensively studied, let alone their complex structure-activity relationships.

Research frontiers

To uncover the dynamic progress and identify the pharmacological metabolites of natural products is one of the hotspots in development of traditional Chinese herbal drugs.

Innovations and breakthroughs

The biotransformation of aesculin was investigated in this study. Human gut bacteria could completely convert aesculin into aesculetin *in vitro*. Six aesculetin metabolites were identified in rat urine, 2 of which were first found. These novel sulfated aesculetin metabolites represent one of the most important modifications of cumarins. As their sulfate groups accumulate negative charges, sulfated cumarins can interact with certain molecular domains that usually have positive charges, resulting in compounds with anti-viral, anti-tumor and anti-oxidative effects. Therefore, these novel derivatives offer new evidence for the biological activities ascribed to aesculin.

Applications

The results of this study can direct the development of anti-viral, anti-tumor and/or anti-oxidative natural herbal drugs, and display a useful design for retrieval of the complex biotranformation process of ceterin natural compounds.

Terminology

LC/ESI-MS stands for Liquid chromatography-electrospray ion trap mass spectrometry; CFU indicates that colony forming units.

Peer review

The authors studied the biotranformation of aesculin by gut bacteria in rats and showed that aesculin had a wide range of biological activities that may have important pharmaceutical applications. Hence, knowledge about its metabolism is essential for understanding its therapeutic effects. The findings are interesting. Aesculin was converted to aesculetin in an *in vitro* bacterial culture system and 6 aesculetin metabolites were identified in rat urine, 2 of which were first observed.

REFERENCES

1 **Kaneko T**, Tahara S, Takabayashi F. Inhibitory effect of natural coumarin compounds, esculetin and esculin, on oxidative DNA damage and formation of aberrant crypt foci and tumors induced by 1,2-dimethylhydrazine in rat colons. *Biol Pharm Bull* 2007; **30**: 2052-2057

2 **Kaneko T**, Tahara S, Takabayashi F, Harada N. Suppression

of 8-oxo-2'-deoxyguanosine formation and carcinogenesis induced by N-nitrosobis (2-oxopropyl)amine in hamsters by esculetin and esculin. *Free Radic Res* 2004; **38**: 839-846

3 **Masamoto Y**, Murata Y, Baba K, Shimoishi Y, Tada M, Takahata K. Inhibitory effects of esculetin on melanin biosynthesis. *Biol Pharm Bull* 2004; **27**: 422-425

4 **Wang CJ**, Hsieh YJ, Chu CY, Lin YL, Tseng TH. Inhibition of cell cycle progression in human leukemia HL-60 cells by esculetin. *Cancer Lett* 2002; **183**: 163-168

5 **Lee BC**, Lee SY, Lee HJ, Sim GS, Kim JH, Kim JH, Cho YH, Lee DH, Pyo HB, Choe TB, Moon DC, Yun YP, Hong JT. Anti-oxidative and photo-protective effects of coumarins isolated from Fraxinus chinensis. *Arch Pharm Res* 2007; **30**: 1293-1301

6 **Yang JY**, Della-Fera MA, Hartzell DL, Nelson-Dooley C, Hausman DB, Baile CA. Esculetin induces apoptosis and inhibits adipogenesis in 3T3-L1 cells. *Obesity* (Silver Spring) 2006; **14**: 1691-1699

7 **Lacy A**, O'Kennedy R. Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer. *Curr Pharm Des* 2004; **10**: 3797-3811

8 **Leung KN**, Leung PY, Kong LP, Leung PK. Immunomodulatory effects of esculetin (6,7-dihydroxycoumarin) on murine lymphocytes and peritoneal macrophages. *Cell Mol Immunol* 2005; **2**: 181-188

9 **Kim BG**, Lee Y, Hur HG, Lim Y, Ahn JH. Production of three O-methhylated esculetins with Escherichia coli expressing O-methyltransferase from poplar. *Biosci Biotechnol Biochem* 2006; **70**: 1269-1272

10 **Duncan SH**, Flint HJ, Stewart CS. Inhibitory activity of gut bacteria against Escherichia coli O157 mediated by dietary plant metabolites. *FEMS Microbiol Lett* 1998; **164**: 283-288

11 **Kanho H**, Yaoya S, Itani T, Nakane T, Kawahara N, Takase Y, Masuda K, Kuroyanagi M. Glucosylation of phenolic compounds by Pharbitis nil hairy roots: I. Glucosylation of coumarin and flavone derivatives. *Biosci Biotechnol Biochem* 2004; **68**: 2032-2039

12 **Gumbinger HG**, Vahlensieck U, Winterhoff H. Metabolism of caffeic acid in the isolated perfused rat liver. *Planta Med* 1993; **59**: 491-493

13 **Stern N**, Nozawa K, Kisch E, Tuck ML, Golub M, Eggena P, Knoll E. Tonic inhibition of renin secretion by the 12 lipoxygenase pathway: augmentation by high salt intake. *Endocrinology* 1996; **137**: 1878-1884

14 **Munoz-Munoz JL**, Garcia-Molina F, Varon R, Rodriguez-Lopez JN, Garcia-Canovas F, Tudela J. Kinetic characterization of the oxidation of esculetin by polyphenol oxidase and peroxidase. *Biosci Biotechnol Biochem* 2007; **71**: 390-396

15 **Park C**, Jin CY, Kim GY, Choi IW, Kwon TK, Choi BT, Lee SJ, Lee WH, Choi YH. Induction of apoptosis by esculetin in human leukemia U937 cells through activation of JNK and ERK. *Toxicol Appl Pharmacol* 2008; **227**: 219-228

16 **Yang JY**, Della-Fera MA, Baile CA. Esculetin induces mitochondria-mediated apoptosis in 3T3-L1 adipocytes. *Apoptosis* 2006; **11**: 1371-1378

17 **Turnbaugh PJ**, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027-1031

18 **Ley RE**, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006; **444**: 1022-1023

19 **Gill SR**, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science* 2006; **312**: 1355-1359

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CASE REPORT

A case of Noonan syndrome and Whipple's disease in the same patient

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Abstract

We report the first known case of both Noonan syndrome and Whipple's disease occurring in the same patient. A 36-year-old female with history of Noonan syndrome developed fatigue, anorexia, arthritis of the knees and hands with a diffuse hyperpigmented rash, night sweats, and an unintentional fifteen pound weight loss over 4 mo. Small bowel enteroscopy demonstrated mild edematous yellowish mucosa without friability. Random small bowel biopsies revealed extensive periodic acid-Schiff positive material within the foamy macrophages. She was treated with a 12 mo course of trimethoprim-sulfamethoxazole DS with clinical improvement to baseline status.

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Key words: Whipple's disease; Noonan syndrome; *Tropheryma whipplei*; Periodic acid-schiff stain; *PTPN11* gene

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INTRODUCTION

We report the first known case of both Noonan syndrome and Whipple's disease (WD) occurring in the same patient. Noonan syndrome is an autosomal dominant genetic disorder that causes abnormal development, originally labeled as the "male Turner syndrome." It affects at least 1 in 2500 male and female children and is thought to be due to a genetic mutation of *PTPN11* gene, first discovered in 2001. Signs and symptoms include webbing of the neck, changes in the sternum (pectus excavatum), facial abnormailities (low-set or abnormally shaped ears, ptosis, hypertelorism, epicanthal folds, antimongoloid palpebral slant, micrognathia), cubitus vulgaris, congenital heart disease (especially pulmonary stenosis, and/or atrial septal defect), and variable hearing loss. Mild mental retardation is present in approximately 25% of cases. WD is a rare systemic infection caused by a non-acid fast gram positive bacillus, *Tropheryma whipplei* (*T. whipplei*). Thus far fewer than 1000 reported cases have been described with an annual incidence of approximately 30 cases per year since 1980. In 1907, Whipple first reported this syndrome in an original case report. Invasion or uptake of the bacillus is widespread throughout the body, including the intestinal epithelium, macrophages, capillary and lymphatic endothelium, colon, liver, brain, heart, lung, synovium, kidney, bone marrow, and skin. Advancement in diagnosis has only recently occurred with the first successful culture of *T. whipplei* in the year 2000; nearly a full century after the disease entity was first described.

CASE REPORT

A 36-year-old female with history of Noonan syndrome developed fatigue, anorexia, arthritis of the knees and hands with a diffuse rash, night sweats, and an

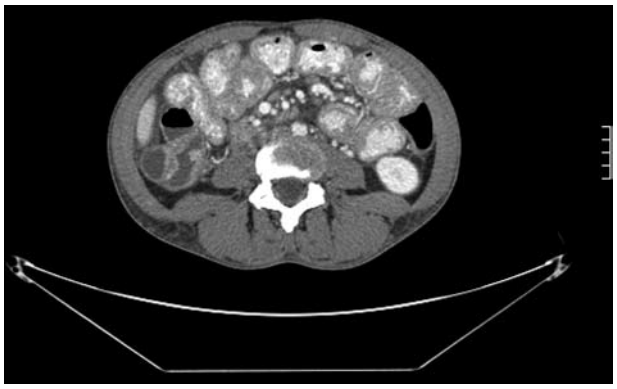

Figure 1 Computerized tomography scan image displaying diffuse lymphadenopathy and small bowel wall thickening.

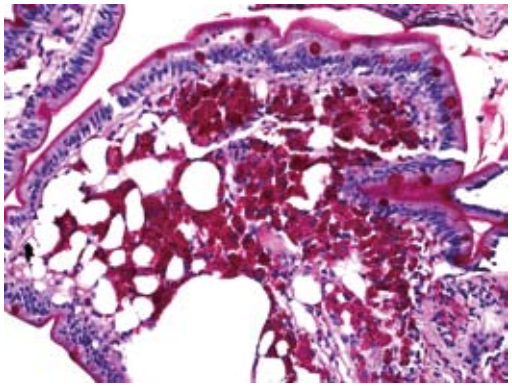

Figure 2 A periodic acid-Schiff (PAS) stain showing extensive PAS positive material within the foamy macrophages (small intestinal biopsy).

unintentional fifteen pound weight loss over the past 4 mo. One year prior, she had an endoscopic workup performed for iron deficiency, which was significant for erosive esophagitis. Of note, ileal and duodenal biopsies were histologically normal. Prior to this consultation, she had recently seen a rheumatologist and was placed on prednisone and Plaquenil for arthritis thought to be of rheumatoid origin. She then developed an episode of acute right lower quadrant abdominal pain and an abdominal computerized tomography (CT) scan was ordered. This revealed lymphadenopathy and diffuse thickening of the small bowel (Figure 1). The patient was then referred for gastroenterological consultation at which time her chief complaints were fatigue, arthralgias, and anorexia. She denied abdominal pain, diarrhea, hematochezia, fevers, chills, nausea, or vomiting.

She was 5'2" feet tall and weighed 117 pounds. Physical examination was significant for arthritis of the knees and hands with mild diffuse hyperpigmented skin. Laboratory tests were significant for sedimentation rate of 33, hemoglobin 9.6, and a white blood cell count of 10.4 without a shift. At this time, concern for lymphoma warranted further investigation. Thus, enteroscopy was performed in May 2006. The only significant endoscopic findings were a mild edematous yellowish mucosa without friability in the distal duodenum and jejunum. Random small bowel biopsies revealed intestinal mucosa in which the lamina propria was markedly expanded due to an infiltrate of histiocytic-appearing cells with foamy cytoplasm and a periodic acid-Schiff (PAS) stain showing extensive PAS positive material within the foamy macrophages; AFB stain was negative (Figure 2). These findings were consistent with a diagnosis of WD. Due to the patient's inability to psychologically tolerate intravenous therapy and an allergy to penicillin, she was placed on an oral regimen of trimethoprim-sulfamethoxazole double-strength twice daily for a 12 mo course.

At follow up in December 2006, she had a remarkable clinical improvement and returned to her baseline clinical status. That is, she had a marked improvement in weight and complete resolution of anemia, arthritis, rash, and bowel wall thickening and lymphadenopathy on CT scan.

DISCUSSION

We report the first known case of both Noonan syndrome and WD occurring in the same patient. Noonan syndrome is an autosomal dominant genetic disorder that causes abnormal development, originally labeled as the "male Turner syndrome." It affects at least 1 in 2500 male and female children and is thought to be due to a genetic mutation of *PTPN11* gene, first discovered in 2001.

WD is a rare systemic infection caused by a non-acid fast gram positive bacillus, *T. whipplei*. Thus far, fewer than 1000 reported cases have been described[1], with an annual incidence of approximately 30 cases per year since 1980. In postmortem studies, the frequency of the disease is less than 0.1%[2]. In 1907 Whipple first reported this syndrome in an original case report[3]. The ability to diagnose WD was advanced in 1949 with PAS staining which identified granules within macrophages that likely represented degenerating bacterial forms[4]. Invasion or uptake of the bacillus is widespread throughout the body, including the intestinal epithelium, macrophages, capillary and lymphatic endothelium, colon, liver, brain, heart, lung, synovium, kidney, bone marrow, and skin. The chronic, insidious nature of WD may at least be in part due to the long doubling time of 17 d. Advancement in diagnosis has only recently occurred with the first successful culture of *T. whipplei* in the year 2000; nearly a full century after the disease entity was first described. One year later, in 2001, the first phenotypic characterization of the Whipple bacillus occurred, resulting in the renaming of the bacterium to *T. whipplei*[5].

Clinical manifestations include four cardinal findings: arthralgias, weight loss, diarrhea, and abdominal pain. Neurological involvement (dementia, supranuclear ophthalmoplegia, nystagmus, and myoclonus) has been recognized in up to 40% of patients, either as initial manifestations or during the course of the disease. Less common symptoms include fever and skin

hyperpigmentation. There may also be symptoms or signs related to cardiac disease (dyspnea, pericarditis, culture-negative endocarditis), pleuropulmonary disease (pleural effusions) or mucocutaneous disease.

Traditionally, the disease has been described in two stages: a prodromal stage and a much later steady-state stage. The time between these stages varies; however, it typically averages 6 years[6]. The prodromal stage is predominantly characterized by nonspecific symptoms including arthralgias and fatigue. More specific findings such as weight loss, diarrhea, and multi organ involvement occur in the steady state stage. Approximately 15% of patients with WD do not present with classic signs and symptoms of the disease; therefore, the diagnosis should be considered in a broad range of clinical scenarios[7,8]. Furthermore, immunosuppressed patients can display a more rapid progression of the disease[9,10].

The clinical manifestations of the disease are believed to be caused by infiltration of *T. whipplei* into various tissues. The patient's immune system reacts by incorporating the bacteria into tissue macrophages. Serum studies typically have presented nonspecific findings, therefore biopsy of the appropriate tissue is essential for diagnosis. Tissue samples from the small bowel show expanded villi containing PAS staining macrophages. After the discovery of PAS staining in 1949, the detection of bacteria in macrophages in 1961 further contributed to our understanding of WD[4,11,12]. However, the only specific diagnostic tests for WD include determining the presence of *T. whipplei* DNA through molecular amplification of the 16S rRNA of *T. whipplei* by polymerase-chain-reaction (PCR) and cell culture of the organism[13-15]. Subsequently, this finding led to electron microscopy, which in turn led to DNA testing for *T. whipplei*. Most recently, in 2003 the full sequencing of two genomes from two different strains of *T. whipplei* has been reported[6,16,17].

In conjunction with clinical signs and symptoms, endoscopy is an important diagnostic modality in WD. Endoscopic examination of the postbulbar region of the duodenum and jejunum includes findings consisting of pale yellow and shaggy mucosa alternating with eroded, erythematous, or mildly friable mucosa[18]. Several biopsy samples should be studied because the lesions can be sparse and focal[6].

In the era before routine access to antibiotics, WD was a universally fatal disease. The discovery of antibiotics has resulted in antibiotics as the mainstay of therapy for WD. Treatment regimens are tailored for severity of disease, however, lack of well-controlled trials limits recommendations to reports of experience. The first reported efficacy of antibiotic treatment with chloramphenicol occurred in 1952[19]. With the progressive discovery of safer antibiotics, tetracycline at one time was the mainstay of therapy for many years. However, a comprehensive review by Keinath *et al*[20] subsequently revealed an overall relapse rate of 35% with a high CNS relapse rate among patients treated primarily with tetracycline[21,22]. As a result, the current standard of therapy includes an initial phase of intravenous antibiotics known to penetrate the blood brain barrier followed by 12 mo of oral maintenance treatment. A typical course often includes ceftriaxone or penicillin for two weeks followed by trimethoprim-sulfamethoxazole double strength twice per day for one year. Depending on allergies to medications, antibiotics can be substituted as needed.

REFERENCES

1 **Roberts IM**. Whipple disease. Available from: URL: http://www.emedicine.com/med/topic2409.htm
2 **Enzinger FM**, Helwig EB. Whipple's disease: a review of the literature and report fifteen patients. *Virchows Arch Pathol Anat Physiol Klin Med* 1963; **336**: 238-269
3 **Swartz MN**. Whipple's disease--past, present, and future. *N Engl J Med* 2000; **342**: 648-650
4 **Black-Schaffer B**. The tinctoral demonstration of a glycoprotein in Whipple's disease. *Proc Soc Exp Biol Med* 1949; **72**: 225-227
5 **La Scola B**, Fenollar F, Fournier PE, Altwegg M, Mallet MN, Raoult D. Description of Tropheryma whipplei gen. nov., sp. nov., the Whipple's disease bacillus. *Int J Syst Evol Microbiol* 2001; **51**: 1471-1479
6 **Fenollar F**, Puéchal X, Raoult D. Whipple's disease. *N Engl J Med* 2007; **356**: 55-66
7 **Misbah SA**, Mapstone NP. Whipple's disease revisited. *J Clin Pathol* 2000; **53**: 750-755
8 **Durand DV**, Lecomte C, Cathébras P, Rousset H, Godeau P. Whipple disease. Clinical review of 52 cases. The SNFMI Research Group on Whipple Disease. Société Nationale Française de Médecine Interne. *Medicine* (Baltimore) 1997; **76**: 170-184
9 **Gerard A**, Sarrot-Reynauld F, Liozon E, Cathebras P, Besson G, Robin C, Vighetto A, Mosnier JF, Durieu I, Vital Durand D, Rousset H. Neurologic presentation of Whipple disease: report of 12 cases and review of the literature. *Medicine* (Baltimore) 2002; **81**: 443-457
10 **Mahnel R**, Kalt A, Ring S, Stallmach A, Strober W, Marth T. Immunosuppressive therapy in Whipple's disease patients is associated with the appearance of gastrointestinal manifestations. *Am J Gastroenterol* 2005; **100**: 1167-1173
11 **Chears WC Jr**, Ashworth CT. Electron microscopic study of the intestinal mucosa in Whipple's disease. Demonstration of encapsulated bacilliform bodies in the lesion. *Gastroenterology* 1961; **41**: 129-138
12 **Yardley JH**, Hendrix TR. Combined electron and light microscopy in Whipple's disease. Demonstration of "bacillary bodies" in the intestine. *Bull Johns Hopkins Hosp* 1961; **109**: 80-98
13 **Wilson KH**, Blitchington R, Frothingham R, Wilson JA. Phylogeny of the Whipple's-disease-associated bacterium. *Lancet* 1991; **338**: 474-475
14 **Relman DA**, Schmidt TM, MacDermott RP, Falkow S. Identification of the uncultured bacillus of Whipple's disease. *N Engl J Med* 1992; **327**: 293-301
15 **Raoult D**, Birg ML, La Scola B, Fournier PE, Enea M, Lepidi H, Roux V, Piette JC, Vandenesch F, Vital-Durand D, Marrie TJ. Cultivation of the bacillus of Whipple's disease. *N Engl J Med* 2000; **342**: 620-625
16 **Bentley SD**, Maiwald M, Murphy LD, Pallen MJ, Yeats CA, Dover LG, Norbertczak HT, Besra GS, Quail MA, Harris DE, von Herbay A, Goble A, Rutter S, Squares R, Squares S, Barrell BG, Parkhill J, Relman DA. Sequencing and analysis of the genome of the Whipple's disease bacterium

Tropheryma whipplei. *Lancet* 2003; **361**: 637-644
17 **Raoult D**, Ogata H, Audic S, Robert C, Suhre K, Drancourt M, Claverie JM. Tropheryma whipplei Twist: a human pathogenic Actinobacteria with a reduced genome. *Genome Res* 2003; **13**: 1800-1809
18 **Marth T**, Raoult D. Whipple's disease. *Lancet* 2003; **361**: 239-246
19 **Paulley JW**. A case of Whipple's disease (intestinal lipodystrophy). *Gastroenterology* 1952; **22**: 128-133
20 **Keinath RD**, Merrell DE, Vlietstra R, Dobbins WO 3rd. Antibiotic treatment and relapse in Whipple's disease. Long-term follow-up of 88 patients. *Gastroenterology* 1985; **88**: 1867-1873
21 **Durand DV**, Lecomte C, Cathébras P, Rousset H, Godeau P. Whipple disease. Clinical review of 52 cases. The SNFMI Research Group on Whipple Disease. Société Nationale Française de Médecine Interne. *Medicine* (Baltimore) 1997; **76**: 170-184
22 **Fleming JL**, Wiesner RH, Shorter RG. Whipple's disease: clinical, biochemical, and histopathologic features and assessment of treatment in 29 patients. *Mayo Clin Proc* 1988; **63**: 539-551

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CASE REPORT

Successful isolation of *Helicobacter pylori* after prolonged incubation from a patient with failed eradication therapy

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Abstract

Helicobacter pylori (*H pylori*), a gastric pathogen, is a major cause of chronic gastritis and peptic ulcer disease, and is an important risk factor for the development of gastric malignancies. Culture of the bacterium from gastric biopsy is essential for the determination of drug resistance of *H pylori*. However, the isolation rates of *H pylori* from infected individuals vary from 23.5% to 97% due to a number of factors such as biopsy preparation, cultural environment, medium and the method adopted. In the present case, we found that a prolonged incubation period of up to 19 d allowed successful isolation of *H pylori* from a patient who received triple therapy that failed to eradicate the bacterium.

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Key words: *Helicobacter pylori*; Isolation; Eradication

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a gastric pathogen, which is present in approximately half of the world's population. It is a major cause of chronic gastritis and peptic ulcer disease, and is an important risk factor for the development of gastric malignancies[1-4]. Although accurate non-invasive methods such as the urea breath test, the stool antigen test, and serology are available, biopsy-based invasive techniques, including the rapid urease test, histology and culture, are required to confirm the infection. Moreover, culture of the bacterium from gastric biopsy is essential for the determination of drug resistance of *H pylori* and thus for the subsequent treatment strategy after failed eradication therapy. However, the isolation rates of *H pylori* from infected individuals vary from 23.5% to 97%[5,6] due to a number of factors, such as biopsy preparation, cultural environment, medium and the method adopted. The duration of incubation for isolation of *H pylori* has been recommended to be 2 to 7 d. Here we reported our observation that a prolonged incubation period of up to 19 d allowed successful isolation of *H pylori* from a patient who received triple therapy that failed to eradicate the bacterium.

CASE REPORT

A patient (female, 59 years old) with an *H pylori* positive duodenal ulcer received two consecutive trials of 7-d triple regimens in a regional hospital. The regimen consisted of Metronidazole, Clarithromycin, and Cimetidine. Four weeks after the second trial, the patient was still positive for a ^{13}C urea breath test. She then came to Beijing for a solution. To obtain the drug resistance profile of the *H pylori* strain, the patient underwent an upper gastrointestinal endoscopy, and four biopsy specimens were taken from gastric antrum. The biopsies were placed directly into transport medium at room temperature and processed for culture within 2 h. Biopsy samples were smeared on *H pylori* selective Dent Columbia agar plates (Oxoid Ltd., London, England) supplemented with 8% sheep blood (Hengzhaoxiang Science & Technology Co., Beijing), and incubated in a microaerophilic environment (5% O_2, 10% CO_2, and 85% N_2) at 37℃. The plates were scheduled to be checked on days 3, 6, 8, and 10. However, there was no colony growing after 10 d. We decided to incubate the plates further, and check the plates every 3 d. On day 19, one suspected *H pylori*-like colony appeared

on one of the plates. This colony was subcultured in non-selective 2 Columbia agar plates containing 8% sheep blood in the same environment mentioned above, for three days. The isolate was confirmed to be *H pylori* based on its typical colony morphology, negative Gram's stain, and positive urease, catalase, and oxidase tests.

The antimicrobial susceptibilities of the isolate to metronidazole, clarithromycin, amoxicillin, ampicillin, levofloxacin, and rifampicin were determined by the E-test (AB Biodisk, Solna, Sweden). Briefly, a bacterial suspension (2 McFarland standard) was prepared with brain heart infusion (Oxoid Ltd., London, England) containing 10% heat-treated serum[7]. After the bacterial suspension was swabbed onto the entire Columbia plates, sterile E-test strips impregnated with the above antibiotics were placed on the agar surface of corresponding plates. Minimal inhibitory concentraction (MICs) were determined according to the manufacturer' s instructions after three to four days of incubation. The isolate exhibited high-level resistance to metronidazole (MIC > 256 μg/mL) and clarithromycin (MIC > 48 μg/mL), but was susceptible to amoxicillin, ampicillin, levofloxacin, and rifampicin (all MIC < 0.016 μg/mL), which explained the failure of the triple regimens containing metronidazole and clarithromycin.

DISCUSSION

Culture has been considered the "gold standard" in confirmation of the diagnosis of *H pylori* infection. Moreover, the isolation and identification of strains is important for the investigation of profiles of bacterial virulence and, particularly, drug resistance. Due to the gradually rising prevalence of *H pylori* resistance to many antibiotics commonly used in triple regimens, the determination of antibiotic susceptibility of individual isolates is of particular importance. However, primary isolation of *H pylori* from gastric biopsies is rather demanding, and is affected widely by the culture conditions in addition to the biopsy-related factors[8]. Our report also indicates that a prolonged incubation is necessary for some strains, especially those enduring hostile environment or a period of antibiotic force. It was reported that a longer incubation of 11 d is helpful for isolating *H pylori* strains from long-term-frozen specimens[9], but this is the first report of the bacteria recovered after 19 d incubation. Indeed, the isolate requiring 19 d recovery later exhibited normal growth characteristics of *H pylori* strains when compared to another strain, NCTC11637, indicating its unusually long incubation requirement was a temporary predicament.

It has been demonstrated *in vitro* that *H pylori* cells can transform from a cultivatable spiral-shaped form to a non-cultivatable coccoid form, in which the recovery of the bacterium is very difficult by routine culture methods[10]. We would propose that during the period of eradication therapy, some organisms transform into the so-called "uncultivatable form" with the propagation being stopped under the antimicrobial pressure in the local environment. However, these organisms, which may have been selectively resistant to the used antimicrobials, survive, possibly with some suppressed metabolic activities[11]. Once released from the medication at the end of the trial, these organisms gradually restore their normal growing features after prolonged incubation in an optimal environment and eventually become cultivatable. Therefore, these "uncultivatable form" organisms might contribute, at least partially, to treatment failures and the development of antimicrobial resistance. In the meantime, we suggest that a new *H pylori* culture after a first attempt to eradicate *H pylori* needs to be postponed, probably by four weeks or even longer. It is noticeable that the patient was positive for the ^{13}C urea breath test four weeks after completion of treatment, indicating that there are a number of organisms that are able to produce urease activities after release from antimicrobial pressure after four weeks. The coccoid *H pylori* can produce urease, though at a decreased level[12], suggesting its potential pathogenicity.

REFERENCES

1 **Dunn BE**, Cohen H, Blaser MJ. Helicobacter pylori. *Clin Microbiol Rev* 1997; **10**: 720-741
2 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131
3 Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. *Gut* 2001; **49**: 347-353
4 **Stolte M**, Bayerdorffer E, Morgner A, Alpen B, Wundisch T, Thiede C, Neubauer A. Helicobacter and gastric MALT lymphoma. *Gut* 2002; **50** Suppl 3: III19-III24
5 **Fresnadillo Martinez MJ**, Rodriguez Rincon M, Blazquez de Castro AM, Garcia Sanchez E, Garcia Sanchez JE, Trujillano Martin I, Cordero Sanchez M, Alvarez Alvarez P, Paz Bouza J, Garcia-Rodriguez JA. Comparative evaluation of selective and nonselective media for primary isolation of Helicobacter pylori from gastric biopsies. *Helicobacter* 1997; **2**: 36-39
6 **Heep M**, Scheibl K, Degrell A, Lehn N. Transport and storage of fresh and frozen gastric biopsy specimens for optimal recovery of Helicobacter pylori. *J Clin Microbiol* 1999; **37**: 3764-3766
7 **Shibayama K**, Nagasawa M, Ando T, Minami M, Wachino J, Suzuki S, Arakawa Y. Usefulness of adult bovine serum for Helicobacter pylori culture media. *J Clin Microbiol* 2006; **44**: 4255-4257
8 **van der Hulst RW**, Verheul SB, Weel JF, Gerrits Y, ten Kate FJ, Dankert J, Tytgat GN. Effect of specimen collection techniques, transport media, and incubation of cultures on the detection rate of Helicobacter pylori. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 211-215
9 **Boyanova L**. Influence of transport conditions and media on Helicobacter pylori isolation. *J Med Microbiol* 2003; **52**: 1129-1130
10 **Shahamat M**, Alavi M, Watts JE, Gonzalez JM, Sowers KR, Maeder DW, Robb FT. Development of two PCR-based techniques for detecting helical and coccoid forms of Helicobacter pylori. *J Clin Microbiol* 2004; **42**: 3613-3619
11 **Nilsson HO**, Blom J, Abu-Al-Soud W, Ljungh A A, Andersen LP, Wadstrom T. Effect of cold starvation, acid stress, and nutrients on metabolic activity of Helicobacter pylori. *Appl Environ Microbiol* 2002; **68**: 11-19
12 **She FF**, Su DH, Lin JY, Zhou LY. Virulence and potential pathogenicity of coccoid Helicobacter pylori induced by antibiotics. *World J Gastroenterol* 2001; **7**: 254-258

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LETTERS TO THE EDITOR

DNA-guided hepatitis B treatment: Viral load is insufficient with few exceptions

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Abstract

In DNA-guided hepatitis B treatment, viral load is insufficient, and requires other viral markers for treatment of hepatitis B patients as in patients with acute exacerbation of chronic hepatitis B, end-stage renal disease on dialysis, human immunodeficiency virus co-infected patients. There are exceptions to this rule: a residual level hepatitis B virus (HBV) DNA at 24 wk predicts beneficial outcome and reduced resistance at 1 year. The genotypic viral resistance to antiviral agents and occult HBV infection can be determined by HBV-DNA levels.

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Key words: DNA; Hepatitis B; Viral load

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TO THE EDITOR

We read with interest the article "DNA-guided hepatitis B treatment: viral load is essential, but not sufficient" by Barcena Marugan *et al*[1]. We agree that viral load is essential but requires other viral marker for treatment of hepatitis B patient.

Patients with exacerbation of chronic hepatitis B, requiring treatment, can be differentiated from acute hepatitis B based on hepatitis B virus (HBV) DNA[2], but the sensitivity and specificity increase with addition of IgM anti-HBc. Low or undetectable DNA levels were seen in acute hepatitis[3], whereas HBV DNA levels became detectable during reactivation of chronic hepatitis[4]. Kumar *et al*[5] in their study showed a low level of HBV DNA (< 0.5 pg/mL) in about 96% of patients with acute infection, as opposed to 13% in those with exacerbation of chronic hepatitis. The sensitivity and specificity of low levels of HBV DNA for identifying an acute infection are 96% and 86.6%, respectively, which increase to 100% and 97.9% respectively with high titers of IgM anti-HBc.

Tong *et al*[6] applied the four criteria (European Association for the Study of the Liver, a treatment algorithm by an independent panel of hepatologists in the United States, an Asian-Pacific consensus statement and the practice guidelines from the American Association for the study of liver disease) to treat 369 HBsAg-positive patients with antiviral therapy. Using these criteria for antiviral therapy as stated by the guidelines, only 20%-60% of hepatocellular carcinoma (HCC) patients and 27%-70% of patients who died of non-HCC were identified for antiviral therapy. If the criteria were broadened with baseline serum albumin 3.5 gm/dL or less or platelet counts of 130 000 mm^3 or less, 89%-100% of deaths from non-HCC liver-related complications and 96%-100% HCC patients would be identified for antiviral therapy.

In patients with end-stage renal disease on dialysis with HBV infection, it remains very difficult to predict the severity and outcome of liver disease based on the HBV-DNA level *per se*[7]. Liver biopsy appears to be the only definitive and reliable means to establish the activity of liver disease in patients on dialysis. It is recommended before starting antiviral therapy and undergoing kidney transplantation. Weisberg *et al*[8] have shown that the estimated 5-year survival rates in patients with end stage renal disease , chronic persistent hepatitis, chronic active hepatitis and chronic active hepatitis with cirrhosis due to hepatitis B are 97%, 86% and 55%, respectively.

In human immunodeficiency virus (HIV) infected patients with HBV, there is an increased risk of cirrhosis,

end-stage liver disease and death from liver disease, especially in patients with a low CD4 cell count or concomitant alcohol use[9]. The treatment of HBV patients co-infected with HIV depends on HBV-DNA levels, histological evidence of active and /or advanced disease (Metavir > A2 and/or ⩾ F2) and CD4 counts whether < or ⩾ 500/mm^3. So, HBV-DNA levels cannot be used alone in co-infected patients with HIV. A CD4 count < 500/mm^3 requires HAART regimen including tenofovir and lamivudine or emtricitabine. A CD4 count ⩾ 500 mm^3 can be treated with entecavir, interferon or adefovir[10].

HBV-DNA load is essential but not sufficient and has few exceptions. Keeffe *et al*[11] showed that complete virologic response (no detectable residual HBV DNA) at 24 wk in patients on anti-viral drugs, and the likelihood of HBeAg sero-conversion and maintenance of an undetectable level of HBV DNA are high, and resistance unlikely occurs. So the residual level HBV DNA at 24 wk can be used as a predictor of beneficial outcome and reduced resistance at 1 year.

The genotypic viral resistance to antiviral agents can be determined by ⩾ 1 $\log_{10}$ IU/mL increase in serum HBV DNA. Virological breakthrough or secondary antiviral treatment failure is usually defined as reappearance or ⩾ 1 $\log_{10}$ IU/mL increase after initial lack of detection or initial ⩾ 1 $\log_{10}$ IU/mL reduction of serum HBV DNA[12]. Virological breakthrough is usually followed by biochemical response[13]. So, a change of serum HBV DNA can be an earliest predictor of viral resistance to antiviral agents. All patients commencing antiviral therapy should have quantitative HBV DNA measurements at baseline and three months after starting therapy[14]. It helps identify response and primary treatment failure in patients on lamivudine.

Occult HBV infection is defined as the detection of HBV-DNA in the serum or liver tissue of patients with negative hepatitis surface antigen[15]. Occult HBV infection has low HBV DNA levels less than 10 000 in the serum and 0.01-0.1 copy per liver cell[16]. The likelihood of antiviral therapy benefit is low as most patients with occult HBV infection have very low levels of HBV DNA. Serum HBV DNA levels fluctuate in cryptic HBV carriers, repeating the HBV test over time is a useful tool in identifying the occult HBV status[17].

REFERENCES

1 **Barcena Marugan R**, Garcia Garzon S. DNA-guided hepatitis B treatment, viral load is essential, but not sufficient. *World J Gastroenterol* 2009; **15**: 423-430

2 **Orenbuch-Harroch E**, Levy L, Ben-Chetrit E. Acute hepatitis B or exacerbation of chronic hepatitis B-that is the question. *World J Gastroenterol* 2008; **14**: 7133-7137

3 **Webster GJ**, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 2000; **32**: 1117-1124

4 **Gayno S**, Marcellin P, Loriot MA, Martinot-Peignoux M, Levy P, Erlinger S, Benhamou JP. Detection of serum HBV-DNA by polymerase chain reaction (PCR) in patients before reactivation of chronic hepatitis B. *J Hepatol* 1992; **14**: 357-360

5 **Kumar M**, Jain S, Sharma BC, Sarin SK. Differentiating acute hepatitis B from the first episode of symptomatic exacerbation of chronic hepatitis B. *Dig Dis Sci* 2006; **51**: 594-599

6 **Tong MJ**, Hsien C, Hsu L, Sun HE, Blatt LM. Treatment recommendations for chronic hepatitis B: an evaluation of current guidelines based on a natural history study in the United States. *Hepatology* 2008; **48**: 1070-1078

7 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225-1241

8 **Weissberg JI**, Andres LL, Smith CI, Weick S, Nichols JE, Garcia G, Robinson WS, Merigan TC, Gregory PB. Survival in chronic hepatitis B. An analysis of 379 patients. *Ann Intern Med* 1984; **101**: 613-616

9 **Thio CL**, Seaberg EC, Skolasky R Jr, Phair J, Visscher B, Muñoz A, Thomas DL. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet* 2002; **360**: 1921-1926

10 **Koziel MJ**, Peters MG. Viral hepatitis in HIV infection. *N Engl J Med* 2007; **356**: 1445-1454

11 **Keeffe EB**, Zeuzem S, Koff RS, Dieterich DT, Esteban-Mur R, Gane EJ, Jacobson IM, Lim SG, Naoumov N, Marcellin P, Piratvisuth T, Zoulim F. Report of an international workshop: Roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin Gastroenterol Hepatol* 2007; **5**: 890-897

12 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507-539

13 **Papatheodoridis GV**, Dimou E, Laras A, Papadimitropoulos V, Hadziyannis SJ. Course of virologic breakthroughs under long-term lamivudine in HBeAg-negative precore mutant HBV liver disease. *Hepatology* 2002; **36**: 219-226

14 **Locarnini S**, Hatzakis A, Heathcote J, Keeffe EB, Liang TJ, Mutimer D, Pawlotsky JM, Zoulim F. Management of antiviral resistance in patients with chronic hepatitis B. *Antivir Ther* 2004; **9**: 679-693

15 **Conjeevaram HS**, Lok AS. Occult hepatitis B virus infection: a hidden menace? *Hepatology* 2001; **34**: 204-206

16 **Cacciola I**, Pollicino T, Squadrito G, Cerenzia G, Villari D, de Franchis R, Santantonio T, Brancatelli S, Colucci G, Raimondo G. Quantification of intrahepatic hepatitis B virus (HBV) DNA in patients with chronic HBV infection. *Hepatology* 2000; **31**: 507-512

17 **Chen CJ**. Time-dependent events in natural history of occult hepatitis B virus infection: the importance of population-based long-term follow-up study with repeated measurements. *J Hepatol* 2005; **42**: 438-440

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Meetings

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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Author(s) and editor(s)

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Conference proceedings

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Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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