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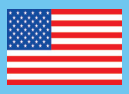

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FIELD OF VISION

Alanine and aspartate aminotransferase and glutamine-cycling pathway: Their roles in pathogenesis of metabolic syndrome

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Abstract

Although new research technologies are constantly used to look either for genes or biomarkers in the prediction of metabolic syndrome (MS), the pathogenesis and pathophysiology of this complex disease remains a major challenge. Interestingly, Cheng *et al* recently investigated possible pathways underlying MS by high-throughput metabolite profiling in two large and well characterized community-based cohorts. The authors explored by liquid chromatography and mass spectrometry the plasma concentrations of 45 distinct metabolites and examined their relation to cardiometabolic risk, and observed that metabolic risk factors such as obesity, insulin resistance (IR), high blood pressure, and dyslipidemia were associated with several metabolites, including branched-chain amino acids, other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. In addition, the authors found a significant association of IR traits with glutamine, glutamate and the glutamine-to-glutamate ratio. These data provide new insight into the pathogenesis of MS-associated phenotypes and introduce a crucial role of glutamine-cycling pathway as prominently involved in the development of metabolic risk. We consider that the hypothesis about the role of abnormal glutamate metabolism in the pathogenesis of the MS is certainly challenging and suggests the critical role of the liver in the global metabolic modulation as glutamate metabolism is linked with aminotransferase reactions. We discuss here the critical role of the "liver metabolism" in the pathogenesis of the MS and IR, and postulate that before fatty liver develops, abnormal levels of liver enzymes, such as alanine and aspartate aminotransferases might reflect high levels of hepatic transamination of amino acids in the liver.

Key words: Alanine; Aspartate; Glutamine; Glutamate; 2-oxoglutarate; Glycolysis; Pyruvate

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INVITED COMMENTARY ON HOT ARTICLES

The metabolic syndrome (MS), a complex disorder associated with several metabolic disturbances and mostly characterized by insulin resistance (IR) in several tissues, results from a complex interplay between genetic and environmental factors[1]. Among the environmental factors, decreased physical activity, increased nutrient availability and over nutrition, play an important role and are also largely considered to be responsible for the modern epidemic of MS-related phenotypes, such as obesity, arterial hypertension and type 2 diabetes (T2D). Moreover, the pathogenesis of IR is strongly associated with the ability of the liver to suppress endogenous glucose production, suggesting that this organ is a key player in the pathophysiology of the MS. Some metabolic disturbances in the hepatic tissue, such as abnormal triglycerides accumulation observed in fatty liver, have been suggested as the trigger events and perhaps the causative factors of IR[2,3]. As such, nonalcoholic fatty liver disease (NAFLD) is now considered to be an additional component of the MS strongly associated with cardiovascular disease (CVD)[1,4-6].

Although significant efforts have been made in the last years and new research technologies are constantly used to look for either genes or biomarkers in the MS prediction, the pathogenesis and pathophysiology of this complex disease remains a major challenge.

We read with great interest the article by Cheng *et al*[7] recently published in *Circulation*. Interestingly, Cheng *et al*[7] investigated possible pathways underlying MS by high-throughput metabolite profiling in two large and well characterized community-based cohorts, including 1015 individuals from the Framingham Heart Study and 746 from the Malmö Diet and Cancer Study. By liquid chromatography and mass spectrometry, the authors explored the plasma concentrations of 45 distinct metabolites and examined their relation to cardiometabolic risk, and found that metabolic risk factors such as obesity, IR, high blood pressure, and dyslipidemia were associated with several metabolites, including branched-chain amino acids (BCAA), other hydrophobic amino acids, tryptophan breakdown products, and nucleotide metabolites. In addition, the authors observed a significant association of IR traits with glutamine, glutamate and the glutamine-to-glutamate ratio in individuals from both cohorts. They described for the first time that a high glutamine-to-glutamate ratio is associated with a lower risk of incident diabetes mellitus. The authors also followed up these findings by a dietary-intervention study in mice, and observed that administration of glutamine led to both increased glucose tolerance and decreased blood pressure[7]. Hence, the authors conclude that individuals with metabolic risk factors have higher circulating concentrations of glutamate and BCAA, and lower concentrations of glutamine, and suggest that glutamate may contribute to the development of the MS. Moreover, the authors observed that circulating levels of BCAA are not only associated with obesity and impaired glucose tolerance but also with dyslipidemia and blood pressure.

What can this metabolomic data tell us about the pathogenesis of MS?

These data open new perspectives about the pathogenesis of MS-associated phenotypes and introduce a crucial role of glutamine-cycling pathway as prominently involved in the development of metabolic risk.

Actually, the role of glutamine-cycle in the regulation of metabolic syndrome-related phenotypes was postulated many years ago, as Hermanussen *et al*[8] showed that chronic hyperglutamatemia may intoxicate arcuate nucleus neurons, thereby disrupting the hypothalamic signaling cascade of leptin action, causing hyperphagia, obesity and hyperleptinaemia. Surprisingly, glutamate has also been associated with metabolic programming and it was postulated that the thrifty phenotype, the epidemiological association between poor fetal and infant growth and the subsequent development of the MS, may be the consequence of fetal hyperglutamatemia[8].

In addition, previous evidences from a human study, including a metabolic profiling performed on 74 obese and 67 lean subjects, identified a cluster of obesity-associated changes in specific amino acid, acylcarnitine, and organic acid metabolites in obese compared to lean subjects that was associated with IR[9]. Newgard *et al*[9] tested the effect of supplementation of high fat diet with BCAA in an experimental study, and showed that this model was associated with decreased levels of circulating α-ketoglutarate and increased levels of glutamate, and speculated that the accumulation of glutamate increases the transamination of pyruvate to alanine, leading to the development of obesity-associated IR. Newgard *et al*[9] in fact extended this reasoning to that the increase in alanine, a highly gluconeogenic amino acid, contributes to the development of glucose intolerance in obesity, as circulating alanine levels are elevated in obese subjects.

Furthermore, a recent human study exploring metabolite predictors of deteriorating glucose tolerance in two Finnish population-based studies consisting of 1873 individuals and reexamination of 618 individuals after 6.5 years in one of the cohorts showed that alterations in BCAA metabolism precede hyperglycemia[10]. In addition, alanine, lactate, and pyruvate were predictive of post-challenge glucose[10]. A candidate gene association study in 9369 non-diabetic or newly diagnosed T2D Finnish men that explored the association of glycemia and 43 genetic risk variants showed that hyperglycemia and a variant of glucokinase (hexokinase 4) regulator (*GCKR*) are associated with the levels of eight amino acids, including alanine, leucine, isoleucine, tyrosine,

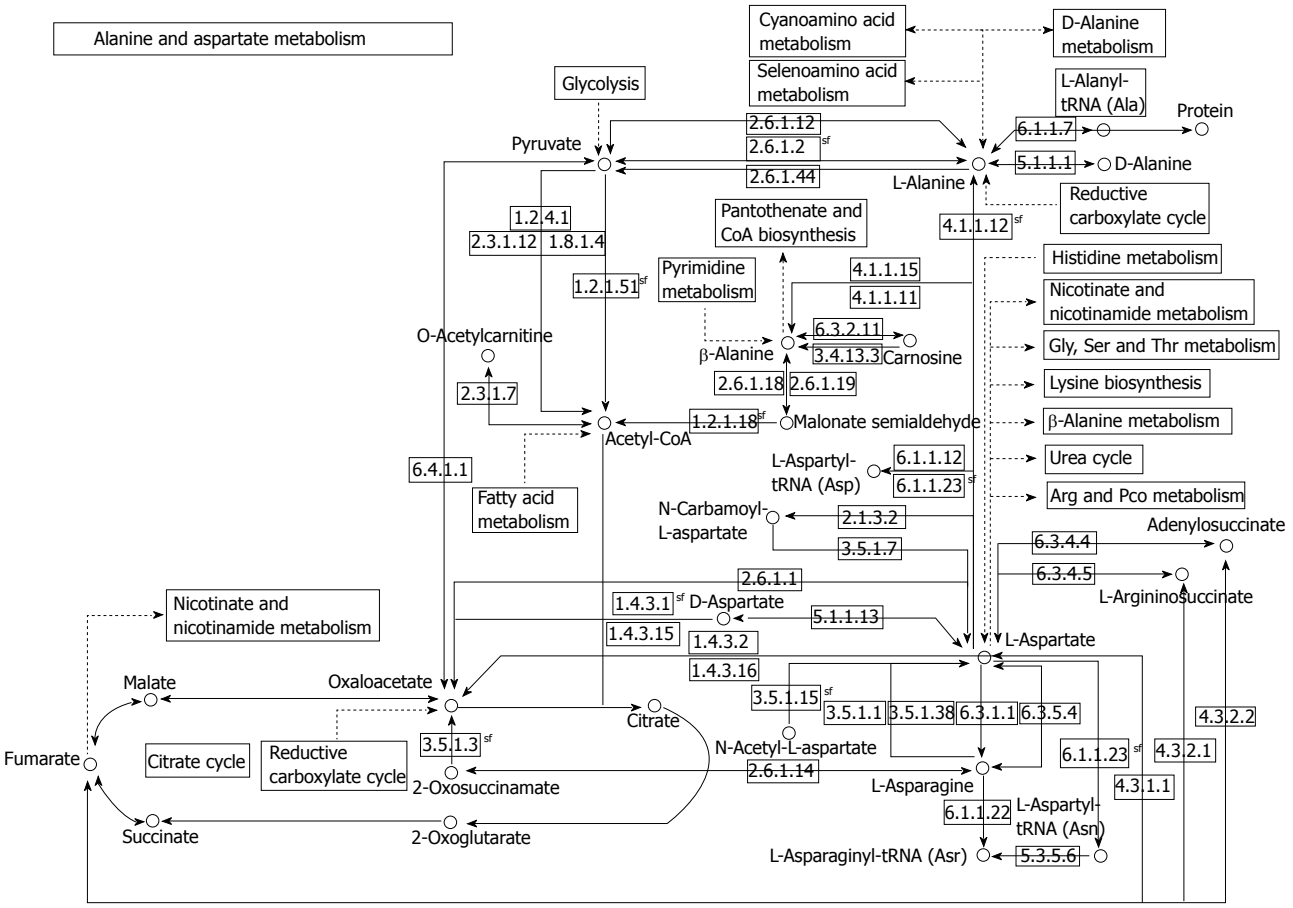

Figure 1 Schematic presentation of the role of aminotransferases alanine and aspartate in metabolic pathways. Alanine and aspartate pathways and their relationship with fatty acid and amino acid metabolism, glycolysis, citrate and urea cycle. Available at: KEGG Metabolic Pathways. http://www.biologie.uni-hamburg.de/b-online/kegg/kegg/Classes/dblinks_java/map/map00252.html.

and glutamine predicted incident T2D in a 4.7-year follow-up[11]. Among the 43 risk variants, only one single nucleotide polymorphism, the glucose-increasing major C allele of rs780094 of GCKR, was significantly associated with decreased levels of alanine and isoleucine and elevated levels of glutamine[11].

The role of the liver in glutamate metabolism: Aminotransferases and glutamate cycle

The hypothesis about the role of abnormal glutamate metabolism in the pathogenesis of the MS is certainly challenging and suggests the critical role of the liver in the global metabolic modulation as glutamate metabolism is linked with aminotransferase reactions. Actually, in the liver, the enzymes of glutamine metabolism critically determine the level of glutamine that is released to circulation[12]. Moreover, glutamate increases the transamination of pyruvate to alanine. In fact, the metabolism of almost all of the amino acids is initiated by aminotransferases, and the transfer of the amino group produces glutamate which may then be substrate of either glutamate dehydrogenase or aspartate aminotransferase[13].

The reactions of transamination mediate the synthesis of aspartate, asparagine, glutamate, and glutamine from ammonia and intermediate of the glycolysis pathway, and allow for the utilization of the carbon atoms from these four amino acids for glucose synthesis under fasting conditions. A short overview of alanine (ALT) and aspartate (AST) aminotransferases is shown in Table 1, and a comprehensive illustration of the AST and ALT pathway is shown in Figure 1.

In summary, ALT not only plays a key role in the intermediary metabolism of glucose and amino acids, but can also be considered as a major contributor to the steady-state glutamate levels as the enzyme can simultaneously catabolize and synthesize glutamine[13].

Biological significance of high ALT and AST levels and cardiovascular risk: Is there any association with altered glutamate metabolism?

Serum activity levels of ALT are routinely used as a biomarker of liver injury caused by drug toxicity, viral infection, alcohol abuse and fatty liver. Nevertheless, several epidemiological studies showed that CVD and the MS are associated with abnormal liver enzymes, such as ALT, even in the absence of liver injury or steatosis. For instance, increased levels of ALT are associated with long-term development of multiple metabolic disorders among participants of the Framingham Offspring Heart Study[14]. Goessling and coworkers also demonstrated that higher ALT levels were significantly associated with an increased risk of T2D and CVD in age-sex adjusted

Table 1 Overview about liver aminotransferases alanine and aspartate

ALT or GPT
Catalyzes the reversible transamination[1] between alanine and 2-oxoglutarate to form pyruvate and glutamate: L-alanine + 2-oxoglutarate = pyruvate + L-glutamate
ALT has both degradative and biosynthetic roles in the glutamate cycling
ALT participates in cellular nitrogen metabolism and also in liver gluconeogenesis starting with precursors transported from skeletal muscles
ALT is present in tissues including liver, kidney, heart, and skeletal muscle.
AST or GOT
Catalyzes the reversible transamination between L-aspartate and 2-oxoglutarate to form oxaloacetate and glutamate: L-alanine + 2-oxoglutarate L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate
Cytosolic AST (GOT 1 catalyzes the reversible reaction of oxaloacetate and glutamate to form aspartate and 2-oxoglutarate (alpha-ketoglutarate)
AST has two isoforms: cytoplasmatic and mitochondrial

[1]Transaminase: A subclass of enzymes that catalyze the transfer of an amino group from a donor (generally an amino acid) to an acceptor (generally 2 keto acid) in a cyclic process using pyridoxal phosphate as a cofactor. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GPT: Glutamate pyruvate transaminase; GOT: Glutamate oxaloacetate transaminase.

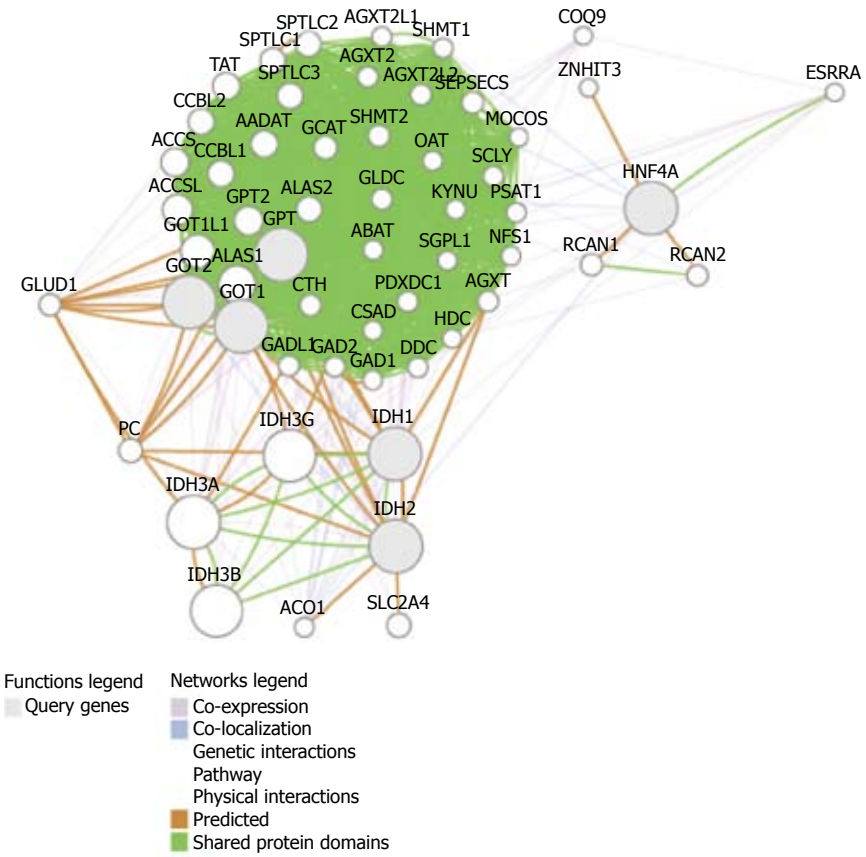

Figure 2 Integrated functional association analysis of protein and genetic interactions on alanine and aspartate. Pathways, co-expression, co-localization, and protein domain similarity were analyzed by the bioinformatics resource GenMANIA (genemania.org) for the 5 candidate genes [alanine also known as glutamate pyruvate transaminase (GPT) and GPT2], aspartate [also known as glutamate oxaloacetate transaminase (GOT)1 and GOT2, (Table 1)], isocitrate dehydrogenases 1 (IDH1), IDH2 and HNF4 (gray circles) and the predicted related genes by systems biology (open circles). List of gene symbol and gene function is shown in Table 2. Predicted functional pathways and Q values are shown in Table 3.

analyses[14]. There was also a significant interaction between body mass index and ALT levels, and the follow-up study of these overweight and obese participants with highest ALT levels for 20 years showed a 30-fold increased risk for developing T2D[14].

The association of ALT with the risk of development MS was also evaluated in 1097 subjects from the population-based cohort of Caucasian men and women (Hoorn Study), and ALT was significantly associated with fasting plasma glucose at follow-up[15]. The 10-year risk of all-cause mortality, fatal and non-fatal CVD in relation to ALT was also assessed in 1439 subjects participating in the Hoorn Study, and the predictive value of ALT for coronary events, seems independent of traditional risk factors[16].

Moreover, findings from the Western Australian Health Department data linkage system, an Australian population-based cohort study, support a strong association between ALT levels and the MS independent of insulin resistance[17].

An overview about the epidemiological evidence of liver enzymes and cardiovascular outcomes was recently published[18].

In spite of the epidemiological evidences mentioned above, the research community is still inconclusive about the pathobiological meaning of the elevated ALT levels and CV risk. In fact, the question of whether abnormalities in ALT levels precede the development of MS, or

Table 2 Candidate gene list (in bold) and 50 predicted genes by systems biology

Symbol	Description	Score
HNF4A	Hepatocyte nuclear factor 4, alpha [source: HGNC symbol; Acc: 5024]	66.77
IDH2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial [source: HGNC symbol; Acc: 5383]	62.04
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble [source: HGNC symbol; Acc: 5382]	61.96
GPT	Glutamic-pyruvate transaminase (alanine aminotransferase) [source: HGNC symbol; Acc: 4552]	58.97
GOT1	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1) [source: HGNC symbol; Acc: 4432]	54.78
GOT2	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) [source: HGNC symbol; Acc: 4433]	54.48
IDH3A	Isocitrate dehydrogenase 3 (NAD+) alpha [source: HGNC symbol; Acc: 5384]	2.68
IDH3G	Isocitrate dehydrogenase 3 (NAD+) gamma [source: HGNC symbol; Acc: 5386]	2.6
IDH3B	Isocitrate dehydrogenase 3 (NAD+) beta [source: HGNC symbol; Acc: 5385]	2.56
ALAS1	Aminolevulinate, delta-, synthase 1 [source: HGNC symbol; Acc: 396]	1.57
GOT1L1	Glutamic-oxaloacetic transaminase 1-like 1 [source: HGNC symbol; Acc: 28487]	1.35
ACCSL	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)-like [source: HGNC symbol; Acc: 34391]	1.14
GPT2	Glutamic pyruvate transaminase (alanine aminotransferase) 2 [source: HGNC symbol; Acc: 18062]	1.03
ACCS	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional) [source: HGNC symbol; Acc: 23989]	0.98
TAT	Tyrosine aminotransferase [source: HGNC symbol; Acc: 11573]	0.93
AADAT	Aminoadipate aminotransferase [source: HGNC symbol; Acc: 17929]	0.88
CCBL1	Cysteine conjugate-beta lyase, cytoplasmic [source: HGNC symbol; Acc: 1564]	0.87
CCBL2	Cysteine conjugate-beta lyase 2 [source: HGNC symbol; Acc: 33238]	0.83
SPTLC3	Serine palmitoyltransferase, long chain base subunit 3 [source: HGNC symbol; Acc: 16253]	0.82
ALAS2	Aminolevulinate, delta-, synthase 2 [source: HGNC symbol; Acc: 397]	0.8
SPTLC2	Serine palmitoyltransferase, long chain base subunit 2 [source: HGNC symbol; Acc: 11278]	0.79
SPTLC1	Serine palmitoyltransferase, long chain base subunit 1 [source: HGNC symbol; Acc: 11277]	0.75
PC	Pyruvate carboxylase [source: HGNC symbol; Acc: 8636]	0.69
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4 [source: HGNC symbol; Acc: 11009]	0.69
GCAT	Glycine C-acetyltransferase [source: HGNC symbol; Acc: 4188]	0.66
RCAN1	Regulator of calcineurin 1 [source: HGNC symbol; Acc: 3040]	0.56
SEPSECS	Sep (O-phosphoserine) tRNA: Sec (selenocysteine) tRNA synthase [source: HGNC symbol; Acc: 30605]	0.56
GLUD1	Glutamate dehydrogenase 1 [source: HGNC symbol; Acc: 4335]	0.55
SHMT2	Serine hydroxymethyltransferase 2 (mitochondrial) [source: HGNC symbol; Acc: 10852]	0.54
CTH	Cystathionase (cystathionine gamma-lyase) [source: HGNC symbol; Acc: 2501]	0.52
AGXT2L2	Alanine-glyoxylate aminotransferase 2-like 2 [source: HGNC symbol; Acc: 28249]	0.49
RCAN2	Regulator of calcineurin 2 [source: HGNC symbol; Acc: 3041]	0.49
AGXT	Alanine-glyoxylate aminotransferase [source: HGNC symbol; Acc: 341]	0.48
GLDC	Glycine dehydrogenase (decarboxylating) [source: HGNC symbol; Acc: 4313]	0.48
GADL1	Glutamate decarboxylase-like 1 [source: HGNC symbol; Acc: 27949]	0.47
PDXDC1	Pyridoxal-dependent decarboxylase domain containing 1 [source: HGNC symbol; Acc: 28995]	0.45
AGXT2	Alanine--glyoxylate aminotransferase 2 [source: HGNC symbol; Acc: 14412]	0.45
GAD2	Glutamate decarboxylase 2 (pancreatic islets and brain, 65 kDa) [source: HGNC symbol; Acc: 4093]	0.43
SCLY	Selenocysteine lyase [source: HGNC symbol; Acc: 18161]	0.43
AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1 [source: HGNC symbol; Acc: 14404]	0.42
ABAT	4-aminobutyrate aminotransferase [source: HGNC symbol; Acc: 23]	0.42
DDC	Dopa decarboxylase (aromatic L-amino acid decarboxylase) [source: HGNC symbol; Acc: 2719]	0.42
KYNU	Kynureninase [source: HGNC symbol; Acc: 6469]	0.41
OAT	Ornithine aminotransferase [source: HGNC symbol; Acc: 8091]	0.4
SHMT1	Serine hydroxymethyltransferase 1 (soluble) [source: HGNC symbol; Acc: 10850]	0.4
PSAT1	Phosphoserine aminotransferase 1 [source: HGNC symbol; Acc: 19129]	0.39
GAD1	Glutamate decarboxylase 1 (brain, 67 kDa) [source: HGNC symbol; Acc: 4092]	0.38
CSAD	Cysteine sulfinic acid decarboxylase [source: HGNC symbol; Acc: 18966]	0.38
NFS1	NFS1 nitrogen fixation 1 homolog (S. cerevisiae) [source: HGNC symbol; Acc: 15910]	0.38
ACO1	Aconitase 1, soluble [source: HGNC symbol; Acc: 117]	0.37
SGPL1	Sphingosine-1-phosphate lyase 1 [source: HGNC symbol; Acc: 10817]	0.36
HDC	Histidine decarboxylase [source: HGNC symbol; Acc: 4855]	0.36
MOCOS	Molybdenum cofactor sulfurase [source: HGNC symbol; Acc: 18234]	0.31
ZNHIT3	Zinc finger, HIT-type containing 3 [source: HGNC symbol; Acc: 12309]	0.3
COQ9	Coenzyme Q9 homolog (S. cerevisiae) [source: HGNC symbol; Acc: 25302]	0.3
ESRRA	Estrogen-related receptor alpha [source: HGNC symbol; Acc: 3471]	0.3

IDH: Isocitrate dehydrogenases; GPT: Glutamate pyruvate transaminase; GOT: Glutamate oxaloacetate transaminase.

whether the MS components themselves can lead to the increase of ALT levels is still unanswered[14]. Hence, the biological mechanisms responsible for the association between liver enzymes and the MS- related phenotypes are still poorly understood, and much of the speculations focus on the putative liver injury associated with fatty liver that frequently coexists with the MS.

The metabolomic data presented by Cheng *et al*[7] not only raised new questions about the role of glutamate-glutamine cycle in the pathogenesis of the MS, but also suggested a dramatic change in the paradigm of the meaning of elevated aminotransferase levels in the context of MS-

Table 3 Gene ontology annotation of predicted biological process

Gene ontology annotation	Q value	Genes in network	Genes in genome
Transaminase activity	4.86E-31	14	16
Transferase activity, transferring nitrogenous groups	6.17E-30	14	18
Mitochondrial matrix	3.10E-15	16	220
Cellular amino acid catabolic process	7.40E-15	12	77
Amine catabolic process	1.13E-14	12	81
Dicarboxylic acid metabolic process	1.75E-14	9	24
Cellular amino acid biosynthetic process	5.90E-13	10	54
Carboxylic acid catabolic process	2.55E-12	12	131
2-oxoglutarate metabolic process	2.55E-12	7	13
Organic acid catabolic process	2.55E-12	12	131
Amine biosynthetic process	8.11E-12	10	72
Glutamate metabolic process	1.08E-10	6	10
Carboxylic acid biosynthetic process	4.53E-10	11	154
Organic acid biosynthetic process	4.53E-10	11	154
Small molecule catabolic process	4.87E-10	12	211
Small molecule biosynthetic process	2.21E-9	12	241
Cofactor metabolic process	2.24E-8	10	163
Vitamin B6 binding	9.53E-8	5	12
Glutamine family amino acid metabolic process	9.53E-8	6	27
Cellular aromatic compound metabolic process	9.53E-8	9	135
Pyridoxal phosphate binding	9.53E-8	5	12
Cofactor binding	6.48E-7	7	69
Aromatic amino acid family catabolic process	1.24E-6	5	19
Coenzyme metabolic process	1.46E-6	8	126
Aromatic compound catabolic process	3.14E-6	5	23
Aromatic amino acid family metabolic process	3.14E-6	5	23
Vitamin binding	8.74E-6	5	28
Indolalkylamine catabolic process	1.08E-5	4	11
Indole-containing compound catabolic process	1.08E-5	4	11
Tryptophan catabolic process	1.08E-5	4	11
Tryptophan metabolic process	1.48E-5	4	12
Indolalkylamine metabolic process	1.48E-5	4	12
Indole-containing compound metabolic process	1.48E-5	4	12
Cellular biogenic amine catabolic process	3.93E-5	4	15
Serine family amino acid metabolic process	5.07E-5	4	16
Acetyl-CoA catabolic process	1.23E-4	4	20
Tricarboxylic acid cycle	1.23E-4	4	20
Coenzyme catabolic process	1.23E-4	4	20
Cofactor catabolic process	3.67E-4	4	26
Aerobic respiration	4.88E-4	4	28
Cellular biogenic amine metabolic process	6.98E-4	5	71
Lyase activity	8.37E-4	5	74
Acetyl-CoA metabolic process	1.01E-3	4	34
Transferase activity, transferring acyl groups other than amino-acyl groups	1.41E-3	5	83
Gluconeogenesis	3.05E-3	4	45
Aspartate family amino acid catabolic process	3.67E-3	3	15
Generation of a signal involved in cell-cell signaling	3.67E-3	6	178
Signal release	3.67E-3	6	178
Transferase activity, transferring acyl groups	3.67E-3	5	103
Sulfur amino acid metabolic process	4.26E-3	3	16
Neurotransmitter secretion	5.20E-3	4	53
Hexose biosynthetic process	5.50E-3	4	54
Water-soluble vitamin metabolic process	6.24E-3	4	56
Monosaccharide biosynthetic process	1.04E-2	4	64
Pteridine-containing compound metabolic process	1.05E-2	3	22
Neurotransmitter transport	1.14E-2	4	66
Carbon-carbon lyase activity	1.48E-2	3	25
Aspartate family amino acid metabolic process	1.48E-2	3	25
Regulation of neurotransmitter levels	1.60E-2	4	73
Sphingolipid metabolic process	1.82E-2	4	76
Alcohol biosynthetic process	1.82E-2	4	76
Pigment biosynthetic process	1.96E-2	3	28
Membrane lipid metabolic process	2.35E-2	4	82
Sphingolipid biosynthetic process	2.35E-2	3	30
Cofactor biosynthetic process	2.53E-2	4	84
Membrane lipid biosynthetic process	2.77E-2	3	32
Cellular modified amino acid metabolic process	2.81E-2	4	87
Cellular carbohydrate biosynthetic process	4.06E-2	4	96
Pigment metabolic process	4.80E-2	3	39
Vitamin metabolic process	4.80E-2	4	101

Q value stands for the *P* value corrected for multiple testing. In addition, number of genes in the network and in the whole genome belonging to the biological process is depicted. The candidate genes are listed in Table 2, which are involved in functional enrichment analysis using the GeneMANIA tool (genemania.org).

related phenotypes. Thus, we speculate that abnormal levels of ALT and AST are associated with a deregulation of normal amino acid metabolism in the liver, including aromatic amino acid, and then special compounds such as glutamate are released into the general circulation. This hypothesis attempts to illustrate the critical role of the "liver metabolism" in the pathogenesis of the MS and IR, and postulates that before the liver becomes fatty, abnormal levels of liver enzymes might reflect high levels of hepatic transamination of amino acids in the organ.

Is there any experimental evidence for this? Stegink *et al*[19] have demonstrated that if a large proportion of glutamate is ingested, portal glutamate increases and this elevation results in increased hepatic glutamate metabolism, leading to release of glucose into systemic circulation, a physiopathogenic event that may perpetuate hyperglycemia.

Systems biology also provides a rational evidence for the association between liver transaminases and the metabolic abnormalities observed by Cheng *et al*[7].

We performed a functional association analysis that included protein and genetic interactions, pathways, co-expression, co-localization, and protein domain similarity using the bioinformatics resource GenMANIA[20]. Interestingly, several genes are regarded as direct "neighbors" of liver transaminases (GPT and GOT1/2, as described in Table 1), but including isocitrate dehydrogenases 1 (IDH1) and 2 (IDH2) and the transcription factor hepatic nuclear factor 4 alpha, because they are involved in the regulation of liver transaminases and glutamine synthetase[21] (Figure 2). Interestingly, the predicted genes (Table 2) belong to pathways that explain most of the findings of Cheng *et al*[7], such as glutamine family amino acid metabolic process, indolalkylamine catabolic process, indole-containing compound catabolic process, tryptophan catabolic process, tryptophan metabolic process, indolalkylamine metabolic process, indole-containing compound metabolic process, cellular biogenic amine catabolic process, among others (Table 3).

Clinical perspective

To conclude, liver transaminases should not be considered as mere biomarkers of liver damage but central players in the pathophysiology of the NAFLD in particular or the MS components in general. Further research has to be done to define whether the elevation of these enzymes is an adaptive or a causative process of the disease.

In particular, because many confounding issues are implied in a pathogenetic relationship with liver, heart and kidney, it is time to look at multi-organ pathogenetic interactions, as recently revised by Bonora *et al*[22].

Finally, mutations in *IDH1* and *IDH2* seem to be critically involved in the generation of certain types of cancers because they create "neoenzymes" that produce 2-hydroxyglutarate, which are required for tumor cell growth from α-ketoglutarate (α-KG)[23]. α-KG is derived from glutamine through its conversion to glutamate by glutaminase. This process may explain the glutamine dependency of the cancer cell growth[24]. Then, it is tempting to speculate that glutamate excess as observed in the MS and NAFLD is an appropriate milieu for cancer development, which may explain the high prevalence of hepatocellular carcinoma in these patients[25], which offers, at the same time, new avenues for its treatment.

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FIELD OF VISION

Diagnostic and therapeutic implications of the association between ferritin level and severity of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD), defined by excessive liver fat deposition related to the metabolic syndrome, is a leading cause of progressive liver disease, for which accurate non-invasive staging systems and effective treatments are still lacking. Evidence has shown that increased ferritin levels are associated with the metabolic insulin resistance syndrome, and higher hepatic iron and fat content. Hyperferritinemia and iron stores have been associated with the severity of liver damage in NAFLD, and iron depletion reduced insulin resistance and liver enzymes. Recently, Kowdley *et al* demonstrated in a multicenter study in 628 adult patients with NAFLD from the NAFLD-clinical research network database with central re-evaluation of liver histology and iron staining that the increased serum ferritin level is an independent predictor of liver damage in patients with NAFLD, and is useful to identify NAFLD patients at risk of non-alcoholic steatohepatitis and advanced fibrosis. These data indicate that incorporation of serum ferritin level may improve the performance of noninvasive scoring of liver damage in patients with NAFLD, and that iron depletion still represents an attractive therapeutic target to prevent the progression of liver damage in these patients.

Key words: Fibrosis; Ferritin; Iron overload; Nonalcoholic fatty liver disease; Steatohepatitis; Steatosis

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INVITED COMMENTARY ON HOT ARTICLES

We read with interest the article by Kowdley *et al*[1] reporting an association between serum ferritin and the severity of liver damage in patients with nonalcoholic fatty liver disease (NAFLD), and strongly recommend it to the readers.

NAFLD is defined by the presence of liver fat deposition related to systemic insulin resistance and the metabolic syndrome[2]. In susceptible individuals, NAFLD is associated with oxidative hepatocellular damage, inflammation, and activation of fibrogenesis, i.e., non-alcoholic steatohepatitis (NASH)[3], with potential progression towards cirrhosis and hepatocellular carcinoma (HCC)[4], whereas simple steatosis is believed to represent a relatively benign condition[5]. Due to the epidemic of obesity and the metabolic syndrome, NAFLD is now the most common liver disease and the leading cause of altered liver enzymes in Western countries[6,7], and it is supposed to become the leading cause of end-stage liver disease, liver transplantation and HCC within the next 10-20 years. Considering the high prevalence of the NAFLD (20%-34% of Western population) two key clinical challenges are expected: the development of noninvasive methods for the diagnosis, staging and follow-up of NASH, and effective treatment strategies counteracting disease progression. Although the gold standard for diagnosis is still liver biopsy, it would be impossible to perform in such a large population at risk, whereas algorithms to predict steatohepatitis and liver fibrosis are still inefficient, need validation, and leave a large grey area[5]. In addition, although promising results have been shown for anti-oxidants[8], no therapeutic trial has yielded convincing results in the progression of liver damage[9], and no pharmacologic therapy is yet approved for NASH.

Over the last years, there has been accumulating evidence about a strong association between hyperferritinemia and mild iron overload unrelated to hereditary hemochromatosis and manifestations of the metabolic syndrome[10-12], including NAFLD[13-18], as recently reviewed by our group[19]. Increased ferritin level was detected in about 30% of unselected patients with NAFLD[20], which has been associated with increased hepatic iron, as determined by histological and radiological assessment, and by quantitative phlebotomy[15,20-22]. However, it is likely that inflammation, cytokines, oxidative and endoplasmic reticulum stress, and the genetic background contribute to ferritin induction by compartmentalization of iron in macrophages in a subset of NAFLD patients without histologically detectable iron stores[19,22-26]. Does hyperferritinemia reflect a subclinical increase in hepatic iron also in this latter subgroup? Very recent data, which have been generated thanks to the availability of magnetic resonance imaging protocols for reliable estimates of tissue iron concentration, confirmed a close link between hepatic iron stores, steatosis, and metabolic diseases. Haap *et al*[27] reported that in healthy non-diabetic subjects, serum ferritin is strongly associated with hepatic iron stores, and hepatic iron stores are independently associated with insulin sensitivity and hepatic fat accumulation. On the other hand, Zheng *et al*[28] reported that in Chinese subjects, subclinical hepatic iron overload is more frequent (60%) in subjects with pre-diabetes and diabetes than in those with normal glucose tolerance, and hepatic iron concentration explains > 40% of glycated hemoglobin variance. Furthermore, in patients with steatosis, body iron stores have been linked to a higher risk of metabolic complications, such as insulin resistance and diabetes[29-31], and to the faster progression of cardiovascular diseases[32-35].

Our group first reported an association between hyperferritinemia, NASH, and the severity of liver damage[13], which was later confirmed by other groups, even if evidence was still controversial[1,14,19,36]. Histological evidence of hepatic iron accumulation has also been associated with an increased risk of fibrosis in large multicenter studies in patients with NAFLD both from Europe and the NASH Clinical Research Network (NASH-CRN) in the United States[15,37], whereas *beta-globin* mutations, the best predictor of parenchymal iron overload in the Mediterranean area, were associated with an almost double risk of severe fibrosis[16]. Furthermore, iron overload has also been associated with HCC in Italian patients with NASH-related cirrhosis[38]. Recent data obtained in animal models are also consistent with a synergistic interaction between liver fat and iron in the pathogenesis of liver damage, which may be related to the induction of iron-dependent cell death (ferroptosis)[39,40]. A schematic presentation of the hypothesized mechanisms underlying hepatic iron accumulation and the role of iron in the progression of liver damage in NAFLD is shown in Figure 1.

Most importantly, iron overload represents also a treatable condition. Experimental evidence suggests that iron depletion induced by chelators induces glucose uptake and utilization in hepatocytes *in vitro* and in the liver *in vivo*, increasing insulin receptor binding activity and signaling[41].

Several reports indicate that iron depletion, most frequently achieved by phlebotomy, may be beneficial in patients with mild iron overload associated with NAFLD. Iron depletion has been first reported to improve insulin sensitivity in a short term in patients with NAFLD with and without increased ferritin levels in two pilot studies in patients with impaired glucose tolerance[42] and normal glucose tolerance[43], and it led to decreased HbA1c levels, heightened insulin secretion and insulin sensitivity in a randomized controlled study in a small number of patients with type 2 diabetes and increased ferritin levels[44]. Both venesection therapy and dietary therapy improved serum ferritin, metabolic parameters and liver function in a controlled study in patients with hepatic iron overload associated with the metabolic syndrome[45]. However, in a matched case-control study in 128 patients with diet-resistant NAFLD, iron depletion reduced insulin resistance more than lifestyle modifications alone, independently of other confounding factors[21]. Finally, in an observational study in 198 NAFLD patients without diabetes with adjustment for propensity score, iron depletion was associated with a higher probability of normalization not only of insulin resistance, but also of liver enzymes during follow-up compared with lifestyle modifications alone[46].

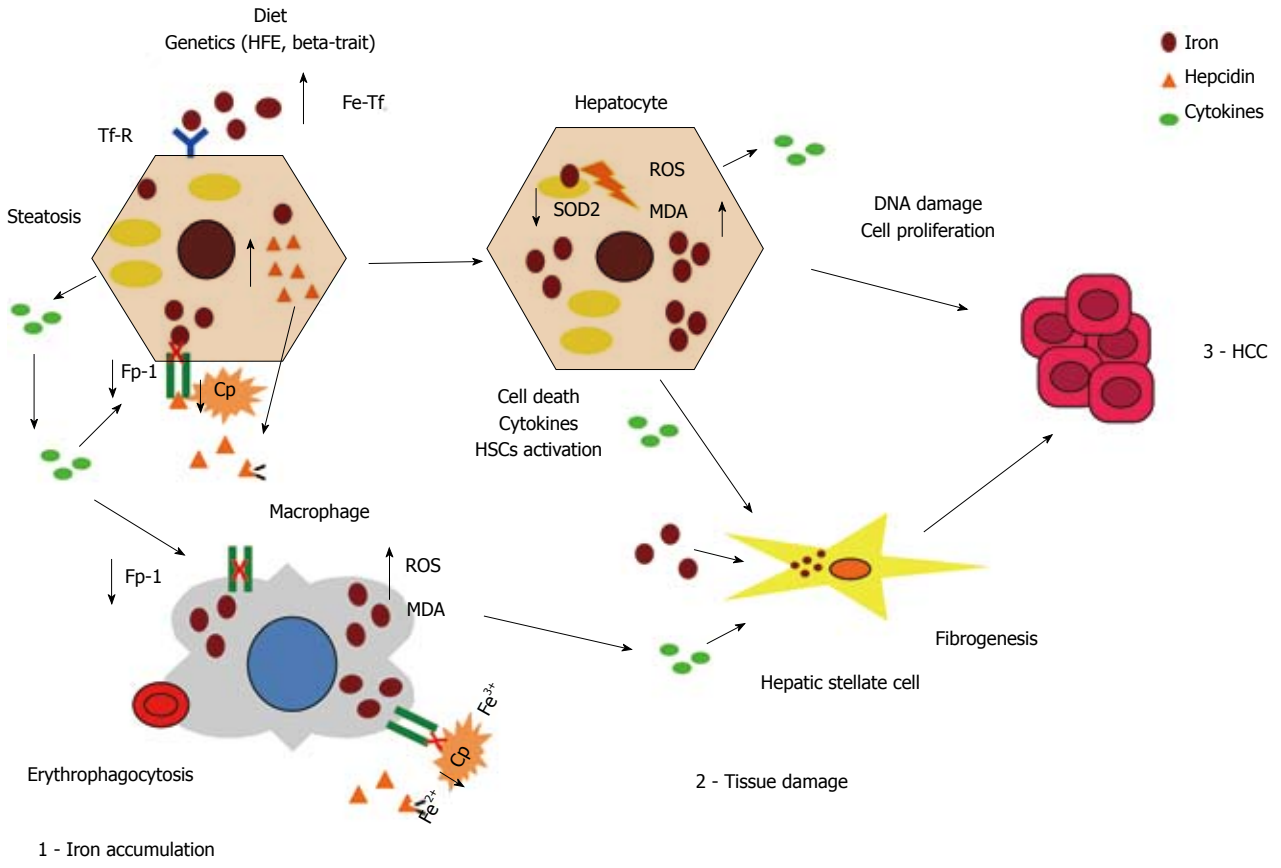

Figure 1 Proposed mechanisms explaining iron induced liver damage associated with steatosis and hepatic iron overload in hepatocytes (brown), macrophages (grey), and hepatic stellate cells (yellow). Cp: Ceruloplasmin; Fe-Tf: Ferric-transferrin; Fp-1: Ferroportin-1; HCC: Hepatocellular carcinoma; HFE: Hemochromatosis gene; HSCs: Hepatic stellate cells; MDA: Malonyl-dialdehyde; ROS: Reactive oxygen species; SOD2: Mn superoxide dismutase; Tf-R: Transferrin receptor. Modified from Dongiovanni *et al*[19].

Within this context, the novel findings reported by Kowdley *et al*[1] provided significant insight into this field. In this study, the authors assessed the relationship between elevated serum ferritin levels (defined as > 1.5 times the upper normal limit) and NAFLD severity in a multicenter study in 628 adult patients with NAFLD from the NAFLD-CRN database with central re-evaluation of liver histology and iron staining. Despite that the upper normal limits were specific for gender, hyperferritinemia was observed more frequently in males (25% *vs* 17%), and was strongly associated with other indices of iron stores including serum iron, transferrin saturation, and hepatic iron staining. Different from what observed in a previous study by our group[15], hyperferritinemia was also associated with alanine and aspartate aminotransferases, but in line with our results[15] with lower platelets, suggesting increased liver fibrosis[47]. Histological features of NAFLD, including steatosis, hepatocellular ballooning, diagnosis of NASH, and fibrosis, were more severe in patients with increased serum ferritin, and at multivariate logistic regression analysis, hyperferritinemia remained significantly associated with advanced hepatic fibrosis [odds ratio (OR): 1.66, 95% CI: 1.05-2.62] and increased NAFLD activity score (OR: 1.99, 95% CI: 1.06-3.75). Based on these results, it is concluded that serum ferritin is associated with hepatic deposition and worsened histological activity in patients with NAFLD, and it is a useful variable to identify NAFLD patients at risk of NASH and advanced fibrosis. An important finding, in view of the previously cited debate about significance of increased ferritin in patients without iron overload, is that hyperferritinemia was associated with enhanced histological activity even in patients without histologically detectable iron deposition.

Although a significant proportion of patients included in the NASH-CRN database had to be excluded due to the lack of ferritin measurement at the time of liver biopsy, important strengths of this study include the analysis of a large multicenter series of patients with histological evaluation, the inclusion of a large percentage of patients with moderate to severe liver fibrosis, and the availability of semiquantitative evaluation of iron stores by Perls' staining and determination of mutations in the hemochromatosis gene of hereditary hemochromatosis in the majority of patients, with concomitant exclusion of patients affected by hereditary hemochromatosis.

Therefore, adding to the previous literature[15,19], this elegant and solid confirmation of the association between hyperferritinemia and severe hepatic fibrosis/NASH has two major implications. The first one and most scientifically grounded is that, whatever its cause (iron overload or altered compartmentalization, inflammation, cellular

stress), in patients with NAFLD, hyperferritinemia is very frequently and strongly associated with liver damage. Since serum ferritin evaluation is widely available in clinical practice and relatively inexpensive, the next step is to test its inclusion in noninvasive prognostic scores of liver damage related to NAFLD to improve their predictive power[1,5,47]. Interestingly, a score incorporating increased ferritin levels, called the "National Association of Fraternal Insurance Counselors score" has already been developed and validated in a large series of Japanese patients, and although the evaluation of a serum marker such as the collagen IV 7S domain is required, that is not easily available outside Japan clinically, it showed a superior predictive power for NASH and severe fibrosis compared with other noninvasive scores[48], and other authors have confirmed the predictive power of ferritin on liver damage in NAFLD[49].

Secondly, but not less important, more clues, that link with increased hepatic iron stores or altered iron compartmentalization in macrophages with steatosis, insulin resistance, and progressive liver disease, are being found[19,50]; and while the results of a small pilot randomized controlled trial evaluating the effect of iron depletion on the progression of histologically evaluated liver damage in patients with NAFLD and increased iron stores (NCT 00658164) are being expected, the accumulated available evidence allows to validate the effect of iron depletion on the prevention of hepatic, metabolic and cardiovascular complications of NAFLD.

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FIELD OF VISION

Tight glycemic control using an artificial endocrine pancreas may play an important role in preventing infection after pancreatic resection

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Abstract

It is well known that perioperative hyperglycemia is the main cause of infectious complications after surgery. To improve perioperative glycemic control, we wish to highlight and comment on an interesting paper published recently by the *Annals of Surgery* entitled: "Early postoperative hyperglycemia is associated with postoperative complications after pancreatoduodenectomy (PD)" by Eshuis *et al.* The authors concluded that early postoperative glucose levels more than 140 mg/dL was significantly associated with complications after PD. Since we recommend that perioperative tight glycemic control (TGC) is an effective method to prevent postoperative complications including surgical site infection after distal, proximal, and total pancreatic resection, we support strongly this conclusion drawn in this article. However, if early postoperative glucose control in patients undergoing PD was administrated by conventional method such as sliding scale approach as described in this article, it is difficult to maintain TGC. Therefore, we introduce a novel perioperative glycemic control using an artificial endocrine pancreas against pancreatogenic diabetes after pancreatic resection including PD.

Key words: Tight glycemic control; Pancreatic resection; Surgical site infection; Artificial endocrine pancreas

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INVITED COMMENTARY ON HOT ARTICLES

It is well known that perioperative hyperglycemia is the main cause of infectious complications after surgery[1]. Figure 1 shows the relationship between hyperglycemia and postoperative infection (POI). Glucose toxicity is caused by surgical stress induced hyperglycemia such as a level of more than 200 mg/dL. Glucose toxicity leads to the leukocyte deficiencies, granulocyte adherence, impaired phagocytosis, delayed chemotaxis, and depressed bactericidal capacity. These abnormalities are the principal causes of POI and they can be improved by appropriate glycemic control[1]. However, optimal blood glucose range to prevent postoperative infectious complications

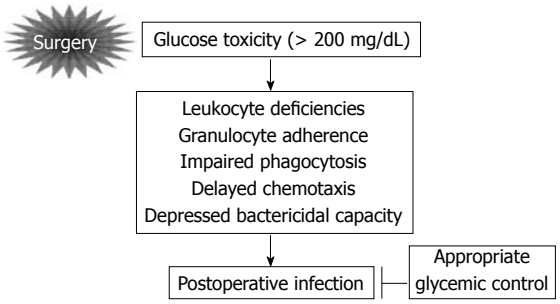

Figure 1 Relationship between hyperglycemia and postoperative infection.

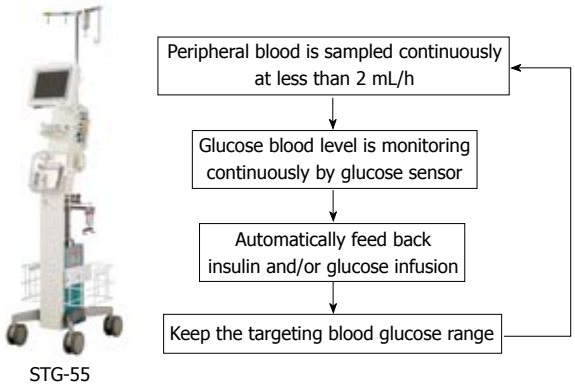

Figure 2 STG-55, a bedside-type artificial endocrine pancreas with closed-loop system.

remains unclear in various surgical settings[1]. To improve the methods for perioperative glycemic control, we would like to recommend an interesting paper published recently on this topic[2]. At the same time, we will introduce a novel perioperative tight glycemic control (TGC) using an artificial pancreas (AP) in patients undergoing pancreatic resection aimed to reduce postoperative infectious complications including surgical site infection (SSI).

We read with great interest the article published in *Annals of Surgery* entitled "Early postoperative hyperglycemia is associated with postoperative complications after pancreatoduodenectomy" by Eshuis *et al*[2]. Among 330 consecutive patients undergoing pancreatoduodenectomy (PD), the average glucose levels were controlled at 135 mg/dL (preoperative), 133 mg/dL (intraoperative) and 142 mg/dL (early postoperative). Pre- and intraoperative glucose levels were not associated with postoperative complications. However, early postoperative glucose levels more than 140 mg/dL was significantly associated with complications after PD[2]. Recent reports indicate that postoperative hyperglycemia increases the risk of postoperative infectious complications and prolongs hospital stay[3-5]. Since we recommend that perioperative TGC is an effective method to prevent postoperative complications including SSI after distal, proximal, and total pancreatic resection[6,7], we support strongly the conclusion drawn in this article[2]. Undoubtedly, this is a significant paper in our understanding of the efficacy of strict perioperative glucose control for patients undergoing PD. However, if early postoperative glucose control in patients undergoing PD was administrated by conventional method such as sliding scale approach as described in this article[2], it seems to be difficult to maintain strict glycemic control with less variability of blood glucose concentration recommended by the authors, including the targeting blood glucose zone of less than 140 mg/dL because pancreatogenic diabetes after pancreatic resection is likely to occur, either hypoglycemia or hyperglycemia, so called brittle diabetes[8,9]. Therefore, we would like to share our opinions regarding more effective and safe TGC against pancreatogenic diabetes after pancreatic resection including PD.

In 2005, we reported that perioperative glycemic control using a closed-loop AP for total pancreatectomized dogs could maintain a stable blood glucose near the normoglycemia[10]. Based on this experimental study, since 2006, we have introduced clinically perioperative glycemic control using an AP[8,11]. As described previously[8,11,12], the Nikkiso Company (Tokyo, Japan) developed a bed-side type AP with closed-loop glycemic control system as STG-22 in conventional device[8] and STG-55 in current device[12] (Figure 2). Detailed mechanisms and characteristics of STG-22 and/or STG-55 were reported previously[8,11,12]. Briefly, peripheral venous blood for glucose monitoring was sampled continuously at less than 2 mL/h. STG-55 (Figure 2)[12] is capable of measuring continuously the blood glucose with its glucose sensor, and automatically infuses insulin and/or glucose to adjust the blood glucose level in accordance with a target blood glucose value, which is the so called closed-loop system[13].

As a result in the clinical surgical settings, our previous report[7] suggested that perioperative TGC using an AP (targeted blood glucose zone of 80-110 mg/dL) in patients undergoing pancreatectomy decreased significantly SSI as compared with that of conventional glycemic control by sliding scale method (targeted blood glucose zone of 150-200 mg/dL). In the sliding scale group, postoperative blood glucose levels rose initially before reaching a plateau of approximately 200 mg/dL between 4 and 6 h after pancreatectomy. The levels remained high for 18 h postoperatively. In the AP group, blood glucose levels reduced steadily, reaching the target zone (80-110 mg/dL) by 6 h after surgery. The total insulin dose administered per patient during the first postoperative 18 h was significantly higher in the AP group (mean ± SD, 107 ± 109 IU) than the sliding scale group (8 ± 6 IU; $P < 0.01$). Neither group showed hypoglycemia[7]. In addition, this novel glycemic control provided for high achievement of targeting blood glucose levels with stable blood glucose concentration[7,8,14]. Moreover, surprisingly, we have never observed occurrence of hypoglycemia less than 40 mg/dL in more than 400 patients undergoing general surgery[12]. Up to dates, we have performed perioperative TGC with target blood glucose levels of 80-110 mg/dL using an AP in more than 100 pancreatectomized patients including more than 50 PD and 10 total pancreatectomies. Of note, every pancreatec-

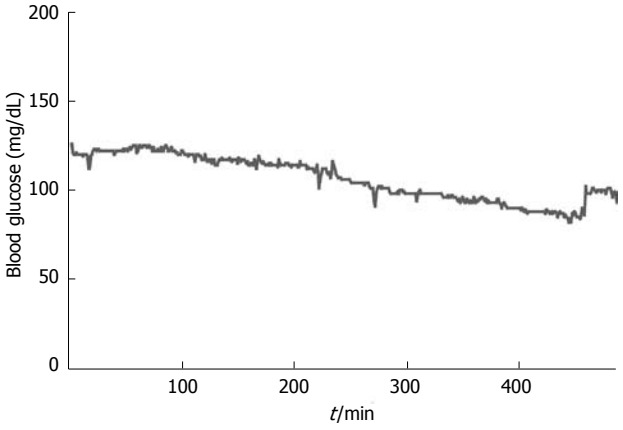

Figure 3 Continuous perioperative blood glucose levels in a case after total pancreatectomy.

tomized patient had stable perioperative blood glucose near the normoglycemia not only without hyperglycemia and/or hypoglycemia but also with less variability of blood glucose concentration, even in a total pancreatectomized patient (Figure 3) who often presented with the most serious pancreatogenic diabetes[9]. Based on these findings from our experimental and clinical studies, we suggest that the AP helps us accomplish an effective and safe perioperative TGC in patients undergoing pancreatic resection.

Interestingly, this article[2] suggests that an early postoperative level of at least more than 140 mg/dL is not recommended for improvement of morbidity after PD. Unfortunately, however, the ideal blood glucose range to reduce mortality and morbidity after PD remains unclear. Therefore, we promote a prospective randomized clinical trial to compared targeted blood glucose range of 80-110 mg/dL group and that of 140-180 mg/dL group in patients undergoing distal, proximal, and total pancreatic resection. Primary end point of this study is incidence of SSI and secondary end points are other postoperative complications and mortality (data not shown). We believe that this novel perioperative glucose control using AP is an easy and a reliable method to maintain targeted blood glucose zone, such as 80-110 mg/dL, 110-140 mg/dL and 140-180 mg/dL, which can be determined freely even in patients undergoing pancreatic resection. In the future perspectives, it is essential to find the optimal blood glucose range to improve morbidity and mortality in patients undergoing pancreatic resection, and then AP will be a useful device to maintain the range.

In conclusion, we suggest that the TGC using an artificial endocrine pancreas with a closed-loop system may play an important role in the effective control of infection after pancreatic resection. This novel perioperative glycemic control will enable us to improve surgical outcome by reducing the postoperative infectious complications due to surgical stress induced hyperglycemia.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Crucial steps in the natural history of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic, progressive and disabling disorders. Over the last few decades, new therapeutic approaches have been introduced which have led not only to a reduction in the mortality rate but also offered the possibility of a favorable modification in the natural history of IBD. The identification of clinical, genetic and serological prognostic factors has permitted a better stratification of the disease, thus allowing the opportunity to indicate the most appropriate therapy. Early treatment with immunosuppressive drugs and biologics has offered the opportunity to change, at least in the short term, the course of the disease by reducing, in a subset of patients with IBD, hospitalization and the need for surgery. In this review, the crucial steps in the natural history of both UC and CD will be discussed, as well as the factors that may change their clinical course. The methodological requirements for high quality studies on the course and prognosis of IBD, the true impact of environmental and dietary factors on the clinical course of IBD, the clinical, serological and genetic predictors of the IBD course (in particular, which of these are relevant and appropriate for use in clinical practice), the impact of the various forms of medical treatment on the IBD complication rate, the role of surgery for IBD in the biologic era, the true magnitude of risk of colorectal cancer associated with IBD, as well as the mortality rate related to IBD will be stressed; all topics that are extensively discussed in separate reviews included in this issue of *World Journal of Gastroenterology*.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Natural history; Clinical course; Complications; Therapy; Surgery; Mortality

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INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic, progressive and relapsing inflammatory disorders of unknown etiology that may cause disability over time. Genetic, environmental and intestinal microbial factors have been reported to play a role in the etiology and pathogenesis of IBD[1]. IBD represents a life-long disorder that may occur at any time from early childhood to late adulthood, although over 80% of cases are cur-

rently diagnosed in the second or third decade of life. UC is characterized by inflammation and ulcerations in the large bowel mucosa and submucosa, whereas CD is a trans-mural inflammatory disorder that may involve various sites of the gastrointestinal tract: in 40%-70% of cases the terminal ileum. Approximately 50% of the patients with IBD present a slight evolutive disease with a low prevalence of relapses, hospitalizations, and complications[2,3]. Other patients have a more severe course and may develop complications that require surgery. In UC, the main complications include toxic megacolon, massive hemorrhage or colon perforation, while strictures and fistulas are uncommon. In CD, intestinal strictures, internal or perianal fistulas or abscesses are frequent, being reported in approximately one-third of patients.

A better understanding of the natural history of this chronic disabling disorder provides valid opportunities: (1) by better defining etiological factors and pathology, it may allow the set up of new strategies of disease prevention; (2) by identifying the relevant clinical subsets in which disease prognosis can be stratified by initial clinical, serological or genetic features, it may be useful in the choice of the most appropriate management of these patients; and (3) by understanding the evolution and the time course of the disease, it may help to define the best end points for clinical trials designed to test drugs modifying disease course[4] (Figure 1).

Questions regarding the natural history and prognosis of IBD are among the most prominent concerns both for patients and clinicians. Unfortunately, most data regarding the course of IBD are obtained from a limited number of cohorts and not all studies focusing on the course and prognosis are of high quality. Adequate knowledge of the methodological requirements of studies focused on prognosis is crucial for interpreting the results of observational studies and for applying these results in clinical practice. In this issue of *World Journal of Gastroenterology* (*WJG*), these methodological aspects have been critically discussed by Modesto *et al*[5]. In particular, they stressed the criteria for an excellent cohort study of prognosis: population-based, use of standard diagnostic criteria for UC and/or CD, start of follow-up already at inception, complete follow-up (80% or more), and use of survival methods to evaluate results.

The crucial steps in the natural history of IBD include the occurrence of lesions, the manifestation and severity of symptoms, the development of complications, the need for surgery, the disability and the mortality (Figure 2). In order to achieve a favorable modification in the disease course, an effective intervention must be carried out at the right time and with a specific therapeutic endpoint (Figure 1). The main outcomes considered include disease activity and relapse, corticosteroid therapy, hospitalizations, complications, surgery, post-operative recurrence, and mortality. More recently, mucosal healing (MH) has been included[6-8]. Early treatment is advisable, before the development of severe bowel damage and impaired functioning[6,8]. There is probably a difference between early disease and long-lasting disease, the control of the disease process being more difficult and unstable in the latter situation[6]. Immunological status of the patients may change over the course of the disease; a stable remission will usually be more difficult to obtain in long-lasting disease and the disease will be more treatment-dependent[6,9].

RISK AND PROGNOSTIC FACTORS

As far as the approach to the treatment of IBD is concerned, it must take into consideration the potential impact of environmental factors (e.g., dietary factors, smoking, psychological stress, drugs and infections) on the clinical course. Clinical, serologic and genetic factors have been reported to be associated with a different clinical course, but their relevance in everyday clinical practice is still controversial[10].

Two reviews in this issue of *WJG* deal with prognostic factors in IBD[11,12].

In the first, Cabré *et al*[11] critically review the role of environmental and dietary factors on the clinical course of IBD. Smoking is the only well established risk and prognostic factor in IBD, with a different impact on CD and UC. In CD, there is consistent evidence that active smoking is a risk factor for post-operative disease recurrence[13,14] but the impact of smoking on the response to therapy is still controversial. In CD patients, the beneficial effect of giving up smoking can be observed; conversely, some patients with refractory UC may even benefit from taking up smoking again[13,15,16]. Based on these observations, therapeutic trials with transdermic nicotine have been performed but with inconclusive results.

The role of dietary habits and dietary manipulation on the clinical course of IBD is far from being well established; therefore, IBD patients are usually encouraged to follow a free diet. As far as concerns the possible therapeutic role of some dietary components, enteral nutrition appears to be effective in CD, particularly in the pediatric population and a low-fat diet seems to be particularly useful even in adult patients[17-20].

With regard to the relationship between psychological stress and IBD, a recent systematic review of 18 prospective studies examining stress as a risk factor for disease exacerbations showed a significant association, and coping behaviors appeared to modulate the effect of stress[21]. Furthermore, it has been reported that approximately 50% of IBD patients had experienced some type of stress; family stress was the most commonly reported form, followed by work or school and financial stress[22].

As far as drugs are concerned, nonsteroidal anti-inflammatory drugs (NSAIDs) are generally considered to potentially affect the IBD course. This concept is not supported by consistent evidence, although in a subset of susceptible patients, NSAID-induced IBD flares appear to occur early after NSAID administration[23]. Current evidence does not support the role of antibiotics and vaccines as a prognostic factor in IBD, albeit antibiotic use is included in the predisposing factors of IBD etiology[24].

Intestinal infections due to enteropathogens have been associated with IBD relapse and the response to therapy[25]. In particular, associated *Clostridium difficile* in-

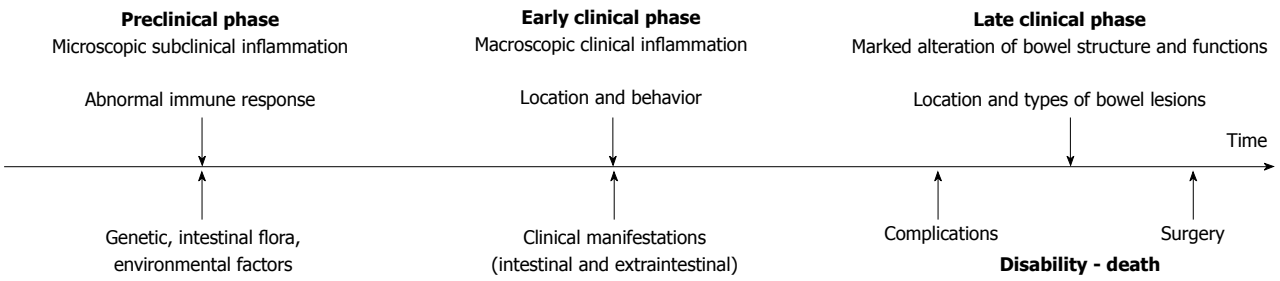

Figure 1 Longitudinal course of inflammatory bowel disease.

fection (which is more frequent in UC than CD) has been reported to have a negative effect on IBD outcome, and to lead to longer hospitalization time, as well as high rates of surgery and a high mortality rate[26]. The role of cytomegalovirus reactivation in the colon of patients with refractory colitis remains to be fully elucidated[27].

In the second paper, Beaugerie *et al*[12] review the role of clinical, serological and genetic predictors of the IBD course and discuss their potential role in clinical practice. This aspect appears to be particularly relevant: there is a need for predictors of a benign or unfavorable clinical course, in order to avoid over-treatment of patients who will experience a mild clinical course and under-treatment of those patients who will experience an aggressive and progressive disorder.

In CD, age under 40 years, perianal disease, ileal lesions and need of steroids at diagnosis have been consistently associated with an unfavorable medium- and long-term clinical course[28-31]. In the post-operative setting, smoking, history of previous resection and severity of early post-operative endoscopic recurrence are the strongest predictors of symptomatic recurrence. In UC, extensive colitis, high disease activity, younger age and female gender are associated with poor outcome in most population-based studies[32-34].

Genetic factors and luminal microbes, besides their role in triggering the IBD, may also be directly, or indirectly, involved in the clinical course of IBD[35,36].

Identifying genetic prognostic factors in IBD is very attractive as they are already present at the onset of the disease and remain stable over time, which is not the case for clinical and serologic parameters. However, despite the growing number of identified susceptibility loci in both CD and UC, only very few have been associated with disease outcomes. The development of genome-wide association scanning techniques has led to the discovery of more than 100 confirmed IBD loci[35-38]. Some of these loci, such as the Th 17 pathway genes (*IL23R*, *IL12B*, *JAK2*, *STAT3*), are shared between CD and UC, others are phenotype-specific (autophagy genes such as *ATG16L1*, *IRGM* and *NOD2* for CD; epithelial barrier genes *HNF4a*, *E-Cadherin*, *LAMB1* and *IL-10* for UC). Variants of some of these genes would be excellent prognostic factors[39].

There is a growing body of evidence proving that in CD, the main *NOD2/CARD15* variants are closely related to ileal disease, a stenosing phenotype, an earlier need for first surgery and a reduced post-operative disease-free interval[10]. All of these findings provide evidence that may encourage the clinical application of *NOD2/CARD15* genotyping both as a marker of CD and as a prognostic factor of the need for early surgery due to stricturing and fibrostenosing disease[10].

Certain genetic factors appear to influence the response to medical treatments. Polymorphisms in multi drug resistant-1, migration inhibitory factor, tumor necrosis factor (TNF) and apoptosis genes have been associated with a higher risk of treatment failure (steroids, cyclosporine, infliximab) in CD and UC[40-45].

Antibodies directed against microbial peptides represent good serological markers that could help in the prediction of the clinical course of IBD[10,46]. Patients with a stronger immune response to microbial peptides are associated with early disease onset of CD, fibrostenosing and penetrating disease, and need for early small bowel surgery[10,46]. In pediatric CD patients, baseline anti-*Saccharomyces cerevisiae* antibody (ASCA) reactivity is associated with earlier complications, relapsing disease and need for additional surgery[10,46]. The frequency of disease complications increases with reactivity to increasing numbers of microbial antigens (ASCA, anti-I2, anti-OmpC, and anti-CBir1). High levels of perinuclear anti-neutrophil antibody are associated with the risk of subsequent chronic pouchitis in UC patients undergoing ileal pouch-anal anastomosis[47].

Overall, these data suggest that serological markers of microbial peptides may be useful predictors of IBD complications. In future, genetic and serological markers will be associated with clinical findings to obtain more reliable and useful predicting tools.

INTESTINAL LESIONS, CLINICAL MANIFESTATIONS AND DISEASE PROGRESSION

Progression of intestinal lesions may range from weeks to decades; however, it can be slowed down, stopped, or reversed spontaneously or by means of medical therapy[2,3] (Figure 2). Superficial mucosal lesions are most prone to heal, whereas deep ulcers or transmural fissures may heal with more difficulty; fibrotic strictures are usually definitive. IBD becomes symptomatic when lesions are extensive or distal, associated with a systemic inflam-

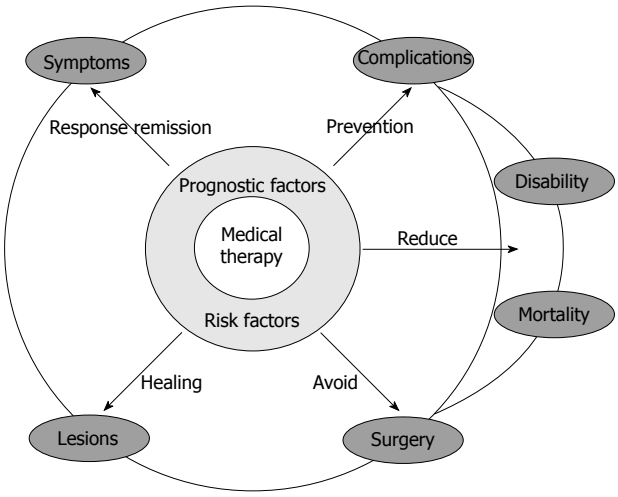

Figure 2 Potential effects of medical therapy on the natural history of inflammatory bowel disease.

matory response, or when associated with local complications such as dilatation (toxic megacolon), massive hemorrhage, strictures, perforation (abscesses and fistulas) and cancer[2,3]. Colorectal lesions usually present more and early symptoms, whereas small bowel lesions may remain latent for several years[2,3]. The disease course is generally characterized by a sequence of flares and remission of varying duration, while approximately one-fifth of these patients undergo a chronic, active, continuous disease course. Abdominal pain, abnormal bowel functions and rectal bleeding are the patients' main complaints that significantly alter their quality of life. Since UC is a pathological disorder that affects the mucosa and submucosa, while CD is a transmural inflammation, the anatomic evolution of the lesions and the disease progression are different, and will therefore be considered separately.

UC involves the rectum and colon and extends proximally in a continuous fashion. Upon presentation, lesions are limited to the rectum (proctitis) in 30%-35% of patients, to the splenic flexure (left-sided colitis) in 30%-45% and to the cecum (pancolitis) in 20%-25%[2,3]. During the course of the disease, after 20 years, the rate of pancolitis may increase reaching 50% of cases. Pancolitis may be associated with inflammation of the terminal ileum ("back wash ileitis"); children do not always have rectal lesions. Mucosal lesions are usually diffuse and superficial, and deep ulcerations are present only in patients with severe disease. Perianal disease may develop in rare cases of UC. Diagnosis may change from UC to CD in 5%-10% of adult patients and in 15%-40% of pediatric patients[2,3,32,48]. UC appears to be particularly severe in younger patients (especially in children), with a higher frequency of flares that do not respond to medical treatment. Severity of flares and their response to therapy vary and are difficult to predict. Disease activity tends to decrease over time, with 40%-50% of patients in prolonged remission and about 30% with active disease. Clinical remission is usually associated with MH[8,49]. Extra-intestinal manifestations are observed in one-third of UC patients[50].

In CD, the lesions can involve any segment of the digestive tract, from the mouth to the anus, but mainly affect the distal ileum and the colon. At the time of diagnosis, approximately 40% of patients present with ileocolonic disease, about 30% have isolated ileal disease, and another 30% have a pure colonic disease[2,3]. Approximately 5%-15% of patients have associated upper gastrointestinal lesions and 20%-30% present perianal lesions[2,3,51,52]. The localization of the lesions changes only minimally over time, with only 10%-15% of patients presenting a change in lesion localization 10 years after diagnosis[53-55].

Although the location remains relatively stable, the clinical behavior of CD shows a dynamic evolution with striking changes over the course of the disease[53-55]. During the first few years of CD, the non-penetrating/non-stricturing (inflammatory) form predominates, whereas most patients develop complications during follow-up and are then classified as having a penetrating or a stricturing disease. These two forms may co-exist in the same patient, since internal fistulae may complicate long-standing intestinal stenosis. Disease evolution is related to lesion localization, the development of complications (abscess, fistula, stricture) being more frequent and rapid when the small bowel is involved, whereas when the disease is localised in the colon, it may remain inflammatory and uncomplicated for many years. There is no relationship between symptoms and progression of the intestinal lesions, since strictures and fistulae may develop for several years with only mild symptoms or, in some cases, without any symptoms at all[3]. Approximately 50% of CD patients have only a slight evolutive disease and, therefore, overtreatment should be avoided[28-30,56]. The remaining patients present a more aggressive and evolutive disease with high rates of relapse, complications, hospitalization and surgery, all conditions that considerably affect the patients' everyday life and long-term projects. For these patients, sustained control of disease activity and progression is clearly warranted. Taken together, these data obviously indicate the need for strategies aimed at interrupting or delaying the natural evolution of this pathological condition. Current treatment options (antibiotics, steroids, immunosuppressive drugs, biological therapies) may relieve the inflammatory symptoms, but do not improve fibrostenotic obstruction[57-63]. The results of medical treatment aimed at stricturing or penetrating CD are poor, since 64% of these patients ultimately require surgery within one year[64]. This situation should be taken into consideration (and discussed with the patient) when planning medical treatment. It is likely that progression to a stricturing or penetrating disease phenotype is an end-stage sequel of CD associated with either irreversible fibrosis or severe inflammation that will not abate despite optimal medical therapy introduced at too late a stage[63].

IMPACT OF MEDICAL THERAPY

The advancements in knowledge of IBD over the past few years have modified the treatment goals. While, in the

past, the aim of medical treatment was an improvement in IBD symptoms, the current objective is to achieve a deep remission, defined, both in UC and CD, as clinical remission [Mayo score for UC activity < 2 and Crohn's disease activity index (CDAI) < 150] with MH (Mayo endoscopic score for UC < 1 and simple endoscopic score for CD < 2) and cessation of steroid administration[6-8,65-67] (Figure 2). Therefore, treatment should modify the course of the disease by avoiding the disabling condition and irreversible tissue damage. The treatment strategy in IBD should, therefore, be tailored according to the risk that each patient runs in developing a disabling disease. In this issue of *WJG*, the review by Reenaers *et al*[68] focuses on the impact of treatment on the natural history of IBD.

Clinical and endoscopic remission is the best result that one can hope to reach and every effort should be made to maintain this condition for as long as possible. Healing of the mucosa, therefore, appears to be an obvious endpoint of treatment. MH can be considered appropriate for UC which is a disease of the mucosa, whereas the term intestinal healing would be more correct for CD which is a transmural disease[67]. Complete assessment of intestinal healing in CD can be obtained only by using both endoscopy and cross-sectional imaging techniques (magnetic resonance, computed tomography, ultrasonography). In CD, it is not uncommon to find healed mucosa covering symptomatic intestinal stenotic segments. Another crucial point is the timing of endoscopic evaluation which was seen to differ (range: 4-52 wk) in studies evaluating the MH[7,8,67]. Many studies have been performed investigating the relationships between clinical symptoms and intestinal lesions but, to date, MH has been evaluated as the primary clinical end point in a single randomized clinical trial[69]. Efficacy of adalimumab (anti-TNFα antibody) for induction and maintenance of MH in 135 adults with moderate-to-severe ileocolonic CD was evaluated in this trial[69]. Twenty-seven percent of patients receiving adalimumab had MH at week 12 (the primary end point) *vs* 13% given placebo ($P = 0.056$). At week 52, rates of MH were 24% and 0%, respectively ($P < 0.001$). Clinical remission rates (CDAI < 150) were 52% for adalimumab and 28% for placebo at week 12 ($P = 0.006$) and 28% and 3%, respectively, at week 52 ($P < 0.001$). Despite the lack of data to confirm an impact of MH induced by anti-TNFs on outcomes in CD, the issue of potential benefits is widely debated. Furthermore, scientific evidence that MH may change the natural course of IBD needs to be proved in long-term studies[6-8,65-67].

The current medical armamentarium for treating moderate-severe IBD consists of corticosteroids, immunosuppressants and biologics (anti-TNFα antibodies), but for most of these medications it is unclear whether treating patients more aggressively will actually slow down disease progression[67,70,71].

In UC, the percentage of patients achieving MH appears to be of the same order for treatment with immunosuppressants (53%-68%)[8,72,73] and biologics (30%-71%)[8,74-76]; albeit, it is obtained more rapidly with the latter agents. Complete restoration of the mucosal architecture may be achieved when acute UC is of short duration and a prompt response to medical therapy has been reached.

In CD, anti-TNFα antibody therapy has been reported, during 54 wk trials, to reduce the need for CD-related hospitalization and surgery[77-80]; however, the duration of these effects is unknown. Although biological therapies have shown disease-modifying characteristics in other pathological conditions, more data are necessary before it can be confirmed whether they can influence the long-term natural history of CD[65,66,81]. There is no doubt that these agents work best when introduced early in the course of the disease, when they could reasonably be expected to change the course of CD. The fact is that they are not: the median duration of disease when this practice is adopted is almost 10 years. It is these patients, who are not frequently found in clinical practice but represent the majority, upon whom attention is focused: new therapeutic agents may add even more delay and ultimately be associated with a higher burden of disease.

It is interesting to note that the overall percentage of CD patients achieving MH with anti-TNFα antibodies (29%-73%)[8,67,77,82-84] is of the same order as that reported with immunosuppressants (35%-73%)[8,67,85-89]. Efficacy of infliximab monotherapy, azathioprine monotherapy, and the two drugs combined in adults with moderate-to-severe CD was evaluated by the Study of Biologic and Immunomodulator Naive Patients in Crohn's Disease (SONIC study)[83]. At week 26, MH had occurred in 43.9% of patients receiving combination therapy, as compared with 30.1% of patients receiving infliximab ($P = 0.06$) and 16.5% of patients receiving azathioprine ($P < 0.001$ for the comparison with combination therapy and $P = 0.02$ for the comparison with infliximab). In a recent prospective comparative study on CD, the MH rate achieved with azathioprine (50%) was not statistically different from that obtained with infliximab (60%)[90]. On the other hand, another recent study showed that only non-complicated inflammatory CD behavior and long-term anti-TNF treatment were associated with a lower risk of the need for surgery, whereas azathioprine only slightly reduced this risk[91].

A crucial point is the timing of commencement of early treatment. In clinical practice, early CD is usually considered as a newly diagnosed case, and this does not always correspond to onset of the early purely inflammatory form of the disease. Approximately 50% of patients already present a stricturing or penetrating disease at the time of diagnosis[55], thus indicating a late disease which is more resistant to treatment both with immunosuppressants and biological agents.

SURGERY

Surgery plays an important role in the management of IBD. Up to 75% of CD patients will require an operation at some point in the course of the disease[92], and, although surgery is not curative, it appears to be the most efficacious treatment in inducing prolonged remission[93].

Therefore, surgery should not be dismissed as the end of the road after all medical options have failed, but should be considered a valid part of the overall management strategy[63]. Nevertheless, in the biological era, avoiding surgery is becoming an emerging and interesting therapeutic endpoint. Considering that biological agents are claimed to induce MH in a large percentage of cases, a substantial reduction in the need for surgery would be expected. Although data from RCTs and observational studies[94] suggest that biological use may reduce the need for surgery in the short term, the real impact of biologics on the lifetime risk of surgery remains to be established. Recent data from population-based cohorts have shown that in the pre-biologic era, the rate of surgery ranged between 27% and 61% within 5 years after diagnosis, and, in the era of anti-TNFα, ranged between 25% and 33%[95], thus suggesting that the need for surgery also remains high in the era of biologics. Moreover, an analysis of secular trends of hospitalization and surgery rates in the United States, from 1990 to 2003, showed stable rates of bowel resection surgery for CD despite advances in treatment[96]. In this issue of *WJG*, de Buck van Overstraeten *et al*[97] discuss the need for surgery in randomized trials as well as the need for surgery in population studies (the real world).

In UC, the cumulative probability of colectomy within 10 years after diagnosis appears to be lower than previously reported[98], but with considerable geographical variations (up to 25% in Denmark and 3.9% in Southern Europe, thus reflecting the different policies in approaching surgery)[99]. In severe UC, colectomy is a life-saving intervention in patients refractory to intravenous steroids; prompt surgery, when necessary, is probably the major determinant of the improved outcome of severe UC in the past 30-40 years[100,101]. During the last 20 years, medical rescue therapy with cyclosporine, and, more recently, with infliximab and tacrolimus, has received growing interest on account of its high efficacy in avoiding colectomy, in the short term, in severe steroid-refractory UC. However, the overall impact of rescue therapies on the outcome of severe UC remains to be defined: the short-term colectomy rate has remained stable over the last 30 years, despite the introduction of cyclosporine[102] and the long-term efficacy of infliximab remains to be defined. In this issue of *WJG*, Dayan *et al*[103] discuss, in detail, the role of surgery in severe UC in the era of medical rescue therapy.

RISK OF COLORECTAL CANCER

There is general consensus that the risk of colorectal cancer (CRC) is increased in IBD. Duration and extent of colitis, persistent inflammatory activity, family history of sporadic CRC and concomitant primary sclerosing cholangitis are well established risk factors. Although CRC risk has been studied more extensively in UC[104], recent data suggest that CD carries a similar risk[105]. However, the exact magnitude of the risk is controversial. In a meta-analysis published in 2001 which included 116 studies, a cumulative probability of CRC in UC of 2% by 10 years, 8% by 20 years, and 18% by 30 years has been reported[104]. These results are in contrast with data from population-based studies from Scandinavia and the United States which report a 30-year cumulative probability of CRC in UC as low as approximately 2% with an overall risk of CRC among UC patients similar to that expected in the general population[106,107].

Although geographical variations in the risk of developing CRC can play a role, differences in the methodology of individual studies (population-based *vs* referral center based) and different clinical approaches in the management of patients and follow-up (cumulative proctocolectomy rate, maintenance treatment with aminosalicylates, close follow-up evaluation of patients and surveillance programs) can explain the high variability in the risk. The exact definition of the risk appears to be crucial when planning "reducing-risk strategies", for example, an endoscopic surveillance program, chemoprevention or both. In fact, the cost-effectiveness of any strategy aimed at reducing the risk of CRC is affected primarily by the baseline risk. Currently there is no strong evidence to support a chemoprophylactic role for 5-aminosalicylic acid, as well as for other drugs used in the treatment of IBD[108-110].

An exhaustive discussion of the molecular biology and all the potential risk factors of IBD-associated CRC is reported by Dyson *et al*[111] in this issue of *WJG*.

MORTALITY

Mortality is the most relevant clinical endpoint in studies focusing on the natural history of a chronic disease. In UC, mortality has continuously decreased over the last 50 years. This time trend probably results from improved medical and surgical management. Data from population-based studies suggest that the overall mortality in UC is not different from that of the background population. However, subgroups of patients, particularly those with extensive disease in the first few years after diagnosis, may be at greater risk of dying[112]. Conversely, CD is associated with a small, but nevertheless significantly increased, risk of death compared to the general population[113].

Although a slight decrease in the standardized mortality ratios has been observed over the last 30 years, this decrease is not statistically significant. This would appear to suggest that the overall prognosis of CD has not really changed despite the improvement in medical and surgical management over the last 30 years.

It will be interesting to see the trend in mortality due to CD in the near future. Preliminary data suggest that in-hospital mortality for CD is reduced in centers with a very large number of admissions for IBD[114], thus suggesting that specialist care could improve outcomes. Besides the reduction in mortality related to the disease, we will, in the near future, also be facing more severe side-effects, including mortality, related to the more aggressive medical treatment.

CONCLUSION

Onset of IBD usually occurs in young adulthood and lasts throughout the patient's life. Despite the enormous progress that has been made in the understanding of these pathological conditions, the etiology remains unknown and no definite cure is yet available[1-3]. The incidence of IBD is increasing worldwide, including also developing countries. UC and CD both have a negative effect on the quality of life and the capacity for work, and, furthermore, increase disability[115]. Disease progression and prognosis have greatly benefited from the use of steroids introduced in the 1950s, immunosuppressants in the 1970s and biological agents in the 1990s. Although these treatments appear to be effective in the management of disease activity in the majority of patients, and to improve the quality of life, it is still not clear whether they are able to modify the natural history of IBD[3,65-67]. There is evidence that new approaches aimed at optimizing immunosuppressants and biological agents by using them as early as possible could prevent disease progression and have a positive effect on the natural history of IBD.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Methodology for high-quality studies on course and prognosis of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBDs) are characterized by a chronic course with an alternation of relapses and remissions. Questions about prognosis are important for the patient who wants to know how the disease will affect his/her life and also for clinicians to make management decisions. Correct selection of the patients is the basis for good methodological studies on the course of IBD. A great proportion of data on the course of IBD is derived from a limited number of cohort studies. Studies help to define the endpoints for clinical trials and to identify subsets of patients in whom the prognosis of the disease can be stratified according to clinical features. Specific scientific requirements for high-quality studies on prognosis are the following: use of inception cohort, description of referral patterns, completeness of follow-up, objective outcome criteria, blind outcome assessment, adjustment for extraneous prognostic factors and statistical issues. We analyzed each of these requirements in studies on IBDs. To date, prospective and population-based cohort studies are the standard for an unbiased assessment of prognosis. A better knowledge of the course of disease of chronic disorders ideally requires: (1) data from population-based studies, to avoid selection bias from referral centers in which patients with a more severe disease are usually treated; (2) inclusion of patients seen at the onset of the disease excluding misdiagnosed cases; and (3) follow-up from the onset of the disease to the end without dropouts.

Key words: Methodology; Inflammatory bowel disease course; Prognosis; Population-based studies; Prospective cohort studies

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are characterized by an alternate course of relapses and remissions. A better knowledge of the course of a chronic disease permits us to answer correctly the questions about the response to therapy, disability, the rate of surgery and mortality, and to identify subsets of patients in whom disease prognosis can be stratified according to clinical features. Finally, studies of the course may increase our knowledge of disease pathology and etiological factors, possibly result-

ing in the prevention of disease.

Until 1970, the course and prognosis of the IBDs were derived initially from tertiary referral centers showing high morbidity and mortality, because they concerned more severe and complicated diseases. Subsequently, several cohort studies have been carried out showing better prognosis than previously described. In fact, for prognosis studies, it is preferable to analyze an unselected group of patients, ensuring the reliability of study results.

Since 1950, drug therapies for IBDs have been introduced, so it is impossible to have any long-term natural history data even if the placebo arms of the clinical trials can be utilized as a source of data on short-term natural history.

According to Sackett *et al*[1], natural history is the course of a disease from its biological onset to its recovery or permanent disability or death. In the spectrum of the course of the disease, we can identify different phases: (1) biological onset, "the initial interaction between man, environment and casual factors"; actually it is not certain what the initial event is; and (2) preclinical phase, interval between biological onset and clinical manifestations. To date, we do not know of any specific markers of disease that allow early diagnosis in this phase. The importance of an early diagnosis is also questionable because of the lack of specific treatment, which can alter the natural history of the disease if initiated in a preclinical phase.

In these first two phases, we deal with natural history because patients have not been treated. However, in all the population studies it is better to use the terminology "course of disease" more than "natural history". The following phases deal with the course of disease: (1) clinical diagnosis that does not correspond to the onset of symptoms (often there is a long gap between the onset of the disease and the time of diagnosis, which represents an important source of bias); and (2) outcome: recovery, permanent disability, mortality. In chronic disease, the interim outcomes (i.e., complications, cancer, impairment of the quality of life, the need of immunosuppression) also represent relevant endpoints in prognostic studies.

In this review, we emphasize the methodological requirements for high-quality studies on the course and prognosis of IBDs. According to Sackett *et al*[1], specific scientific requirements for high-quality studies on prognosis are the following: (1) use of inception cohort; (2) description of referral patterns; (3) completeness of follow-up; (4) objective outcome criteria; (5) blind outcome assessment; (6) adjustment for extraneous prognostic factors; and (7) statistical issues. We analyzed each of these requirements within the studies on IBDs.

INCEPTION COHORT

To evaluate the prognosis of a disease in a cohort of patients, it is important to start the follow-up at a common point; preferably as early as possible in the course of the disease (i.e., onset of symptoms or clinical diagnosis). For this reason, inception cohorts, preferably prospective, now represent the standard design to minimize bias. Bias in cohort studies can create apparent differences when they do not actually exist in nature.

The most frequent selection biases in conducting studies on prognosis are as follows. (1) Prevalence-incidence bias: when mild or asymptomatic cases, for example, proctitis, as well as fatal short disease episodes, such as severe colitis, are missed when studies are performed late in the disease process. It could result in an overestimation of the severity of the disease if the patients with a mild disease are missed, and in a more favorable prognosis if the mortality is not included in the prevalent group; (2) Lead time bias: occurs when the outcomes such as survival, as measured from the time of diagnosis, may be increased not only, because the patients live longer, but because screening permits an early diagnosis (i.e., for the availability of a new diagnostic test). This results in an apparent prolongation of the time to a predefined event (i.e., death, time to surgery or time to relapse), when instead it only results in an earlier diagnosis when compared to traditional methods [i.e., detection of asymptomatic colon cancer during screening endoscopy in patients with ulcerative colitis (UC) could result in an apparent prolongation of survival]; and (3) Length time bias: screening tends to detect a disease that is destined to progress slowly and, therefore, has a good prognosis. Also, advances in diagnostic techniques allow an earlier diagnosis, in an asymptomatic phase of the diseases with less aggressive course. Length time bias occurs when the patients, whose disease is discovered by screening, may also appear to do better, or live longer, than people whose disease is clinically diagnosed with symptoms. For example in patients with IBDs, before the introduction of endoscopy, mild colonic or distal disease, which are often mildly symptomatic, were often missed. This distortion is called technical bias and is related to the length time bias; together with the therapeutic bias (concerning the advance in therapy) it concurs to determine the temporal bias.

DESCRIPTION OF REFERRAL PATTERNS

In a study of prognosis, it is of great importance to use unselected patients to obtain more realistic results and for a wider applicability of study results. However, it is important to describe the referral pattern, which occurs when the characteristics of patients differ between one setting (e.g., primary care) and another setting that includes only referred patients (e.g., secondary or tertiary care). Studies from referral centers include more severe and complicated cases and usually result in poor prognosis.

Another relevant bias is the diagnostic/therapeutic access bias that occurs when studies made between populations with a different access to diagnostic facilities or therapy are compared. For example, in a tertiary center, patients have more opportunities to access biological ther-

apy, allowing a better course of disease in severe patients.

The outcome could also be influenced by the different health insurance or government programs across countries, as is the limit which exists in some countries (i.e., the United Kingdom) on the maximum duration of anti-tumor necrosis factor (TNF) therapy. Few data are available on the relationship between the length of maintenance therapy with anti-TNFα and the natural history of the disease, or with the achievement of mucosal healing, which actually seems to be the main outcome correlated with the maintenance of remission. The best study is the population study in which all the incident cases in a well-defined area are identified and followed up regularly with a clear protocol.

COMPLETENESS OF FOLLOW-UP

According to Sackett *et al*[1], in an accurate study of prognosis, at least 90% of the population should complete follow-up. This statement results from the evidence that a study with many patients lost during follow-up (usually > 20%) leads to distorted results. For example, if patients are lost during follow-up, for poor compliance, it could result in a better prognosis of the cohort. If they are affected by mild disease (like proctitis in UC) and therefore omit control visits, this can result in an overestimation of poor outcomes. However, for any degree of loss during follow-up, the validity of the study could be diminished. In addition to this, the length of follow-up is also important if one is evaluating some specific outcomes like survival. In that case, the follow-up time should be long enough so that about two-thirds of the patients will have suffered the events under study at the end of the observation period. In other cases, when the outcome evaluated is more frequent and rapid in occurrence (i.e., postsurgical recurrence or response to therapy), the follow-up could be shorter.

OBJECTIVE OUTCOME CRITERIA AND BLIND ASSESSMENT

The most important outcomes to assess in a prognosis study are disease activity, intestinal complications, surgery, cancer risk and mortality. One of the most relevant problems in the study of prognosis of IBDs is the difficulty of identifying an objective outcome because of the lack of an agreement in the definition of some important outcome measures that are open to possible differences in the results.

Disease activity

For example, analyzing some of the most important studies of prognosis, the definition of disease activity is variable. Below, we report some examples of this variability in cohort studies of IBDs.

Remission or no activity: (1) In the Copenhagen study[2-7] for Crohn's disease (CD) no activity was defined as no more than two stools per day and no blood or pus in the stools, no abdominal pain and no systemic symptoms such as fever or weight loss; and (2) in the Olmsted County study[8-11], remission or no medication state was defined as a patient who required no medication for CD, excluding antidiarrheals.

Mild disease: (1) In the Copenhagen study[2-7], mild disease activity was defined as ⩾ 2 and ⩽ 4 bowel movements and/or blood or pus in the stools and/or mild abdominal pain less than daily and no systemic symptoms; and (2) in the Olmsted county study[8-11], mild disease was defined according to therapy; a patient with mild disease was a patient on sulfasalazine, 5-acetylsalicylic acid, antibiotics, or topical therapy.

Severe disease: (1) In the Copenhagen study[2-7], moderate/high activity was defined as more than four stools daily and/or blood or pus daily and or abdominal pain either severe or daily, with or without systemic symptoms; and (2) in the Olmsted study[8-11] the authors distinguished severe disease drug responsive and severe disease drug refractory; in the former, they referred to a patient on oral corticosteroids or immunosuppressive therapy lasting > 6 mo, with documented improvements; in the latter definition they included patients on oral corticosteroids or immunosuppressive therapy with no documented improvements within 2 mo for corticosteroids or within 3 mo for immunosuppressive medications.

In another important study, as in the European collaborative study on inflammatory bowel disease[12-17], there was not a clear definition of disease activity; the course of the disease was assessed comparing the activity in a given point during the follow-up with the initial status. Any of these definitions involves subjective judgment and blind outcome assessment, one of the requirements, is not usually feasible and the study results are difficult to compare. Probably the CD activity index[18] and Mayo Clinic score[19] for UC are a more objective outcome to evaluate disease activity or response to therapy in clinical trials, but owing to their complexity, they are not often used in clinical practice.

Complications

Another bias that occurs when collecting data retrospectively, in evaluating intestinal and extraintestinal complications or need of surgery, is referral bias, which occurs when the appearance of complications has triggered the visit. Thus, it is essential that data on the occurrence of complications in IBDs are collected prospectively and in unselected samples, and the diagnostic measures are well defined. It is important to define the diagnostic and therapeutic protocol for complications because a different approach among centers influences the course of the disease. For example, endoscopic dilation is an approach adopted in stricturing postsurgical recurrence in CD in some centers, whereas in others, surgery is the preferred option and this different choice may influence prognosis.

Cancer risk and mortality

In the evaluation of cancer risk, an important concern is represented by the influence of surveillance bias. Surveillance bias, what some texts call detection bias, occurs when one group is followed more closely than another. This could lead to an outcome being diagnosed more often in the more closely followed group, but not because it really occurred more often in that group. Of course, cancer risk is linked to the surgical policy of the single center. A center that proposes early intervention may have a lower risk of cancer in long-term follow-up. It is mandatory that the cancer risk is evaluated in an incident cohort and in a population study. Another relevant requirement is the presence of a cancer registry in the area where the cohort is followed-up.

Another important outcome in prognosis studies is mortality. A recognized method to assess mortality is the calculation of standardized mortality ratio (SMR). SMR is the ratio between the observed number of deaths in a study population and the number of would-be-expected deaths, based on the age- and sex-specific rates in a standard population and the age and sex distribution of the study population. If the ratio of observed/expected deaths is > 1.0, there is said to be "excess deaths" in the study population. It is, however, a very efficient stratification method and also permits one to use retrospective data. The results of studies with good methodological requirements have been summarized in a meta-analysis[20], and give a reliable measure of this outcome correcting for differences among centers. Of course, meta-analysis should include studies with the same methodological standards. Small differences will only be detected if the studied group is very large. At the same time, if the baseline risk of an outcome (i.e., cancer or mortality) is very low, few events in the study population can identify an apparent relevant risk difference (i.e., the risk of Hodgkin lymphoma identified in the Florence cohort[21,22]).

ADJUSTMENT FOR EXTRANEOUS PROGNOSTIC FACTORS

Examining the effects of specific factors on prognosis, it is important to adjust for extraneous variables, for the potential effect of associated factors on the results, thus unmasking a possible erroneous association. These confounding factors can also influence data from different population-based inception cohort studies. Even population-based inception cohorts could be difficult to compare because of the presence of different sources of bias, such as temporal, diagnostic access and therapeutic. Thus, it is important always to give information about the distribution of potential confounding prognostic factors.

In UC, it is important to know the extent of the distribution of the disease (pancolitis, left-side colitis, or proctitis) at diagnosis and the duration of disease because of the known major risk of cancer in pancolitis and in long disease duration.

In CD, many relevant prognostic factors have been identified that should always be included in a multivariate analysis, such as smoking habits, age, site of disease, and extent of disease. Prognostic factors should be evaluated in incident cohorts prospectively.

An example of a possible bias in the evaluation of the prognostic factor is the study by Beaugerie *et al*[23]. Among 1526 patients diagnosed with CD between 1985 and 1998, those operated upon within the first month of the disease, patients with inadequate data, and patients with severe chronic nondigestive disease were excluded. The authors identified age < 40 years, perianal disease, and initial use of steroids as predictive factors for subsequent 5-year disabling. The authors suggested that referral bias could have distorted the results and a further study in a population setting was advocated. Of course, the prognostic model identified in an incident cohort should be applied in another independent cohort (the test sample).

STATISTICAL ISSUES

To date, to evaluate survival in prognosis studies, life-table-based methods have been used to minimize the difficulty in interpreting crude rates deriving from studies with different lengths of follow-up. During a follow-up period, a decrease in the number of patients makes it easier to detect differences in the early stages of follow-up. Some problems could derive from the lack of study power, and caution should be exercised when the effects are examined over different intervals of time. Rare events, such as lymphoma, risk being overestimated because the baseline risk in the general population is low. Finally, it would be desirable that data on the number of patients under observation at a given time are reported as confidence intervals. Cox's proportional hazard analysis is a type of multivariable analysis used when the outcome is the time to obtain the event. When data on important prognostic factors are not available, sensitivity analysis is a useful tool, assuming various degrees of maldistribution between groups, and seeing how it affects the results.

CONCLUSION

The validity of prognosis studies on IBDs is based on the presence of the above-mentioned methodological requirements. An excellent cohort study must fulfill the following criteria: (1) start of follow-up at inception; (2) population-based, or near to population-based; (3) use of standard diagnostic criteria for UC and/or CD; (4) use of survival methods; and (5) complete or near to complete follow-up (≥ 80%).

Better knowledge of the course of chronic disorders ideally requires: (1) data from population-based studies to avoid selection bias from referral centers where patients with more severe disease are usually treated; (2)

Table 1 Population-based prospective and retrospective studies

Study	Population size	Inception period	UC	CD	Surgery UC	Surgery CD	Mortality UC SMR (95% CI)	Mortality CD SMR (95% CI)
Prospective studies								
Copenhagen[2-7], Denmark	550 000	1962-1987	1160	374	24%	61%	1.1 (1-1.2)	1.3 (1.1-1.6)
		1991-1993	89	58	24%	65%	1.5 (0.9-2.5)	2.3 (1.1-4.2)
	1 211 634	2003-2004	326	209	-	-	0.9 (0.3-2.4)	0.8 (0.02-4.2)
		1962-2004	1575	641				
EC-IBD study[12-17]	NA	1991-1993	1379	706	8.70%	40%-55%	1.09 (0.86-1.37)	1.85 (1.3-2.55)
		1991-2004	-	365				
IBSEN[23-28], Norway	970 000	1990-1993	525	225	9.8% (7.4-12.4)	37.9% (31.4-44.4)	Survival 96%	Survival 96%
Retrospective studies								
Stockolm[29-31], Sweden	1 200 000	1955-1984	1547	1251	28.00%	71%	1.37 (1.2-1.54)	1.51 (1.29-1.75)
		1955-2000	-	20 120		78% (15 yr)	(15 yr)	(15 yr)
	1 470 000	1990-2001	-	1389				
Uppsala[32,33], Sweden	1 200 000	1965-1983	2509	1469	-	96% (15 yr)	1.4 (1.2-1.5)	1.6 (1.4-1.9)
Olmsted[8-11], United States	110 000	1940-1993	278	225	49.00%	49%	0.8 (0.6-1)	1.2 (0.9-1.6)
	124 000	1940-2000	372	308				
Leicester[34,35], United Kingdom	930 000	1972-1989	1014	610	-	-	0.9 (0.8-1.1)	0.72 (0.5-1)
Florence[21,22], Italy	650 000	1978-1992	689	231	-	-	0.7 (0.56-0.88)	1.51 (1.06-2.08)
Cardiff[36-41], Wales	280 000	1986-1991	-	105	-	59%	-	
		1992-1997		99		37%		
	NA	1998-2003		137		25%		
		1941-2000		394				1.29 (1.12-1.45)
Leiden[41], The Netherlands	440 000	1979-1983	-	210	-	56% (15 yr)	-	2.23 (1.75-2.85)

UC: Ulcerative colitis; CD: Crohn's disease; SMR: Standardized mortality ratio; EC-IBD study: European collaborative study on inflammatory bowel disease; IBSEN: Inflammatory bowel south-eastern Norway; NA: Not available. Number of cases may vary between various reports from same centers.

inclusion of patients seen at the onset of the disease excluding misdiagnosed cases; and (3) follow-up from the onset of the disease to the end without dropouts.

The more relevant cohort studies are summarized in Table 1 (prospective and retrospective)[2-7,12-17,23-41], which have been followed up for a long period and in which the methodological requirements listed above are satisfied. Two main outcomes are included in the table to show the variation between both types of study, despite the same methodology being adopted. Prospective cohort studies are a more relevant source of information. Although there was wide variation in the rate of surgery, which depends on the therapeutic policy adopted in different areas, mortality was homogeneous in the three main studies.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Clinical, serological and genetic predictors of inflammatory bowel disease course

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Abstract

Patients with extensive or complicated Crohn's disease (CD) at diagnosis should be treated straightaway with immunosuppressive therapy according to the most recent guidelines. In patients with localized and uncomplicated CD at diagnosis, early use of immunosuppressive therapy is debated for preventing disease progression and limiting the disabling clinical impact. In this context, there is a need for predictors of benign or unfavourable subsequent clinical course, in order to avoid over-treating with risky drugs those patients who would have experienced spontaneous mid-term asymptomatic disease without progression towards irreversible intestinal lesions. At diagnosis, an age below 40 years, the presence of perianal lesions and the need for treating the first flare with steroids have been consistently associated with an unfavourable subsequent 5-year or 10-year clinical course. The positive predictive value of unfavourable course in patients with 2 or 3 predictors ranges between 0.75 and 0.95 in population-based and referral centre cohorts. Consequently, the use of these predictors can be integrated into the elements that influence individual decisions. In the CD postoperative context, keeping smoking and history of prior resection are the strongest predictors of disease symptomatic recurrence. However, these clinical predictors alone are not as reliable as severity of early postoperative endoscopic recurrence in clinical practice. In ulcerative colitis (UC), extensive colitis at diagnosis is associated with unfavourable clinical course in the first 5 to 10 years of the disease, and also with long-term colectomy and colorectal inflammation-associated colorectal cancer. In patients with extensive UC at diagnosis, a rapid step-up strategy aiming to achieve sustained deep remission should therefore be considered. At the moment, no reliable serological or genetic predictor of inflammatory bowel disease clinical course has been identified.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Natural history; Predictors; Clinical practice

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INTRODUCTION

The lifelong risk of developing inflammatory bowel disease (IBD) exceeds one percent in industrialized countries[1]. Such diseases are considered as chronic, with a small trend towards spontaneous long-term extinction in ulcerative colitis (UC)[2], but not in Crohn's disease (CD)[3]. Despite an increasing use of immunosuppressive thera-

py, the long-term risk of intestinal resection and permanent ileostomy in CD is approximately 80% and 10%, respectively[1]. In UC, the actuarial risk of colectomy is about one percent per year in population-based cohorts of Northern Europe[4]. To reverse these unfavourable figures that have not significantly changed during the last five decades[5], the early use of aggressive therapy, such as the combination of thiopurines and anti-tumor necrosis factor (TNF) therapy[6], is considered both in CD and UC, with the aim of bringing patients into deep and sustained remission. However, some CD patients present at diagnosis with localized and uncomplicated (no perforation, no stricture) disease, and some UC patients present at diagnosis without disabling symptoms, biological abnormalities or severe endoscopic lesions. In those patients, the early and prolonged use of immunosuppressive therapy, with its associated risk of serious infections[7] and cancers[8,9], is questionable because the spontaneous evolution of the disease could have been benign. The risk of over-treating patients could be reduced in theory with the use of clinical, biological or endoscopic factors present at diagnosis and able to predict the subsequent course of IBD. In CD, there is also a need for predictors after complete resection of intestinal lesions when considering the postoperative preventive use of immunosuppressive therapy for limiting the risk of clinical recurrence. We review here the literature on clinical, serological and genetic predictors of IBD course, and discuss which predictors can be potentially useful in clinical practice.

CD

Clinical predictors of CD course

Prediction at diagnosis of unfavourable 5-year or 10-year clinical course: This question has been specifically studied in two referral centre cohorts[10,11] and two population-based cohorts[12-14], with fixed 5-year or 10-year study period and complete follow-up in most patients (Table 1). Markers of unfavourable CD course were either a single event (first clinical recurrence[14] or surgical operation[12,13]) or the presence of at least one element of a composite definition of disabling disease[10,11]. Whatever the definition, age below 40 years and presence of perianal disease at diagnosis were identified in most studies as predictors of subsequent unfavourable evolution. Need for steroids for treating the first flare and presence of upper gastrointestinal lesions were associated with a poor outcome in two studies. Finally, in two single studies, terminal ileal location and ileo-colonic lesions were predictive of first surgical operation and disabling disease, respectively. Of note, active smoking status, which is an established transient worsening factor of CD course[15,16], was not shown to be an independent predictor of unfavourable short and mid-term CD course in these studies.

Other studies: As a general feature, clinical predictors of unfavourable CD course identified in the above dedicated 5-year and 10-year studies have been confirmed in other studies with various design, follow-up time and endpoints. In the historical national cooperative CD study, perianal disease was an independent predictor of unfavourable outcome among 1084 patients[17]. In a Portuguese referral centre cohort testing CD outcome according to Vienna classification and other clinical markers, earlier age at diagnosis, penetrating lesions (including perianal fistulas and abscesses) and ileo-colonic location were associated with unfavourable outcome[18]. In the Olmstedt population-based study, young age was predictive of surgery, irrespective of smoking status[19]. In the Maastricht population-based cohort, young age, small bowel location and stricturing lesions were predictive of first surgery in 476 patients with a mean follow-up of 7 years[20]. In a referral centre population from Boston, early surgery (within 3 years of diagnosis) was associated with active smoking status, ileal location and need for early steroids in 345 patients followed for at least 3 years[21].

Whether childhood- and adult-onset CD courses differ in terms of severity has been recently questioned[22,23]. Patients with childhood-onset disease tend to have more extensive intestinal involvement, more active disease requiring more immunosuppressive therapy, and more rapid disease progression. By contrast, CD patients with a family history of IBD do not exhibit a more severe disease course in population-based studies[24] as well as in referral centre populations[12]. Finally, erythema nodosum and pyoderma gangrenosum, at diagnosis or later in the disease, are not predictive of more severe CD course[25].

Clinical predictors of long-term CD course: There are very scarce data on predictors of the long-term evolution of CD. In the Saint-Antoine cohort, predictors of 15-year CD course were characterized in 600 patients[1]. Non-severe evolution was defined as clinically inactive disease for greater than 12 years, less than one intestinal resection without permanent stoma and no death. Factors independently associated with a non-severe 15-year clinical course were non-smoking status, rectal sparing, high educational level, older age and longer disease duration.

Clinical predictors of postoperative recurrence: Literature on clinical predictors of postoperative recurrence is abundant and has been extensively reviewed recently[26-28]. In brief, postoperative active smoking status appears in most studies as a strong predictor of postoperative recurrence: the risk of clinical recurrence and re-operation is approximately doubled in smokers[29], with an increase in the risk according to the number of cigarettes smoked per day among smokers[30]. The other independent undisputed predictor of postoperative recurrence is a history of prior resection. The positive predictive value of penetrating disease behaviour, short disease duration prior to surgery, non-colonic disease location, long duration and extensive bowel resection has been evidenced

Table 1 Clinical predictors of unfavourable course of Crohn's disease and ulcerative colitis

Study	Cohort type	No. of patients	Definition or marker of unfavourable course	Independent predictors of unfavourable course, present at diagnosis
Crohn's disease				
Within the first 5 yr after diagnosis				
Beaugerie *et al*[10], 2006	Referral centre (Saint-Antoine)	1188	Disabling disease[1]	Age < 40 yr Perianal disease Need for steroids for treating the first flare
Henriksen *et al*[12], 2007	Population-based (IBSEN)	200	Intestinal resection within the study period	Upper gastrointestinal lesions
Loly *et al*[11], 2008	Referral centre (Liège)	361	Disabling disease[1]	Perianal disease Need for steroids for treating the first flare Ileo-colonic lesions
Within the first 10 yr after diagnosis				
Wolters *et al*[14], 2006	Population-based (EC-IBD)	358	First recurrence	Upper gastrointestinal lesions Age < 40 yr
Solberg *et al*[13], 2007	Population-based (IBSEN)	197	First surgical operation	Age < 40 yr Stricturing and penetrating behaviour[2] (including perianal fistulas and abscesses) Terminal ileal location
Ulcerative colitis				
Within the first 5 yr after diagnosis				
Henriksen *et al*[12], 2007	Population-based (IBSEN)	454	Relapse during the study period	Female gender Younger age
Within the first 10 yr after diagnosis				
Langholz *et al*[2], 1994	Population-based (county of Copenhagen)	1161	Relapsing or chronic active course Colectomy	Less systemic symptoms (fever, weight loss) Extensive colitis High disease activity (including systemic symptoms)
Höie *et al*[56], 2007	Population-based (EC-IBD)	771	Frequent relapse	Female gender Younger age Non-smoking status
Höie *et al*[57], 2007	Population-based (EC-IBD)	781	Colectomy	Extensive colitis
Solberg *et al*[4], 2009	Population-based (IBSEN)	423	Colectomy	Extensive colitis

[1]One or more of the following criteria: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for surgical disease; [2]According to Vienna classification. IBSEN: A population-based inception cohort study; EC-IBD: European collaborative study group of inflammatory bowel disease.

less consistently. Finally, the roles of young age at onset and family history remain controversial.

Serological predictors of CD course

The search for serologic markers in IBD has led to the discovery of specific antibodies. Perinuclear anti-neutrophil antibody (pANCA) is associated with UC or UC-like CD whereas anti-*Saccharomyces cerevisiae* antibody (ASCA, glycan antibody) is mostly associated with CD[31,32]. Three other markers linked to immune response towards bacteria have been identified; antibodies to the *Escherichia coli* outer-membrane porin C (OmpC), the *Pseudomonas fluorescens* CD-related protein [anti-CD related bacterial sequence (I2)] and the CBir1 flagellin[33,34].

Many studies have been performed to assess the predictive value of these serological markers. Reactivity to ASCA, OmpC, anti-I2 and CBir1 has been associated with early disease onset CD, fibrostenosing and penetrating disease and need for early small bowel surgery[34-36]. In paediatric CD patients, baseline ASCA reactivity has been associated with earlier complications, relapsing disease and need for an additional surgery[37]. The frequency of disease complications increases with reactivity to increasing numbers of antigens (ASCA, anti-I2, anti-OmpC, and anti-CBir1)[38]. pANCA has been shown to be associated with less severe disease, UC-like disease and to be negatively associated with small bowel complication[36,39]. ASCA positivity has been associated with CD of the pouch after ileal pouch-anal anastomosis (IPAA)[40].

Genetic predictors of CD course

Before the era of genome-wide association studies, the role of genetic factors in IBD severity had been looked for by comparing familial and sporadic IBD. Although having a relative with IBD increases the risk for CD, the severity of CD is unaffected by family history[24]. A family history of CD increases the risk of subsequent CD after IPAA. Identifying genetic prognostic factors in IBD is a very attractive option as they are already present at the onset of the disease and actually even earlier. Their main advantage is their long-term stability, which is not the case for many other potential predictive factors such as clinical parameters or serologic markers. However, despite a growing number of identified susceptibility loci in both CD and UC[41], only very few have been associated with disease outcome. The presence of *NOD2* polymorphism has been associated with a more aggressive clinical course of CD; i.e., higher risk of intestinal strictures,

Table 2 Validation of the positive predictive value of Saint-Antoine predictors

Study	Cohort type	No. of patients	Prevalence of unfavourable course[2] (%)	Positive predictive value of the presence of 2 or 3 predictors[1] for predicting 5 yr unfavourable clinical course (%)
Reference study				
Beaugerie *et al*[10], 2006	Saint-Antoine referral population	1123	85.2	2 predictors: 91 3 predictors: 93
Validating populations				
Beaugerie *et al*[10], 2006	Independent subsequent sample of the Saint-Antoine referral population	302	85.2	2 predictors: 84 3 predictors: 91
Loly *et al*[11], 2008	Liege referral population	361	57.9	2 or 3 predictors: 67
Beaugerie *et al*[62], 2009	Population-based population (Olmstedt county)	423	74.2	2 or 3 predictors: 74

[1]Age < 40 years and perianal lesions at first presentation, need for steroids for treating the first flare; [2]Unfavourable clinical Crohn's disease course (one or more of the following criteria: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for surgical disease) within the 5 years following diagnosis.

earlier need for first surgery and reduced postoperative disease-free interval[42-44]. Some studies have also tried to identify genetic factors associated with response to treatment. Polymorphisms in multi-drug resistant 1 (*MDR1*), *TNF* and migration inhibitory factor genes have been associated with corticosteroid refractoriness or sensitivity in CD and UC[45-47]. *MDR1* polymorphism has also been associated with a higher risk of cyclosporine failure in patients with steroid-resistant UC[48]. The efficacy of infliximab is partly due to the induction of apoptosis in activated T lymphocytes. A research group in Leuven has analyzed infliximab-treated patients and found polymorphisms in apoptosis genes predicting response to infliximab therapy in luminal and fistulizing Crohn's disease[49]. Other studies suggest that genetic polymorphisms might be useful to predict anti-TNF therapeutic responsiveness in paediatric IBD[50].

Despite their attractiveness, genetic markers will probably never be able to fully predict IBD behaviour and complications, because of the major role of environmental factors in the disease pathogenesis. On the other hand, they could be associated with other factors, such as clinical or microbiological data, in order to build relevant composite predicting tools.

Relevance and potential use of predictors in CD

Saint-Antoine clinical predictors at diagnosis and early use of immunosuppressive therapy: In the Saint-Antoine cohort, patients were considered as having an unfavourable ("disabling") clinical course in the first five years after diagnosis if they met at least one of the following criteria during the study period: more than 2 steroid courses, steroid dependence, hospitalization for disease flare or complication, disabling chronic symptoms, need for immunosuppressive therapy, intestinal resection or surgical operation for perianal disease. Among the 1123 patients studied, the rate of unfavourable course was 85%. By multivariate analysis, initial need for steroid use, age below 40 years and perianal disease at diagnosis were the three independent predictors of subsequent 5-year unfavourable course. The positive predictive value of unfavourable course in patients with two or three predictors was 0.91 and 0.93, respectively.

The Saint-Antoine clinical predictors are the only predictive factors that have been tested subsequently in independent population samples (Table 2). In a subsequent group of 302 patients from the Saint-Antoine centre, the positive predictive value of unfavourable course in patients with two or three predictors was 0.84 and 0.91, respectively[10]. The Saint-Antoine predictors were subsequently evaluated in two independent cohorts, using the same definition of unfavourable CD course as in the reference study. In the Liege referral population, the positive predictive value of unfavourable course in patients with two or three predictors was 0.67[11]. This rate was 0.74 in the Olmstedt population[51]. In conclusion, using the three Saint-Antoine predictors at diagnosis, the prediction of subsequent 5-year unfavourable CD course is accurate in more than two-thirds of patients from various populations. It is plausible that prediction accuracy holds true for the 5 to 10-year CD clinical course, since a strong homology has been demonstrated between the course pattern of CD in the first 3 years of the disease and the 7 subsequent years[3,52].

In the literature, the proportion of patients exhibiting unfavourable clinical course in the first 5 years of the disease, whatever the definition (disabling disease, frequent clinical relapses, chronic disabling symptoms, need for surgery, *etc*.) exceeds 50%, with an expected trend towards higher prevalence in referral centre cohorts than in population-based studies[53]. In clinical practice, patients presenting at diagnosis with irreversible penetrating or stricturing lesions, requiring (or not) immediate operation, can be considered as having complicated disease at diagnosis requiring early aggressive therapy (Figure 1). In the second recent European Crohn's and Colitis Organisation (ECCO) CD consensus, patients with upper gastrointestinal lesions or extensive (> 100 cm) lesions at diagnosis are also considered as having severe disease at presentation which justifies immediate use of immunosuppressive therapy[54]. In the remaining patients, the need for treating moderate to severe flares

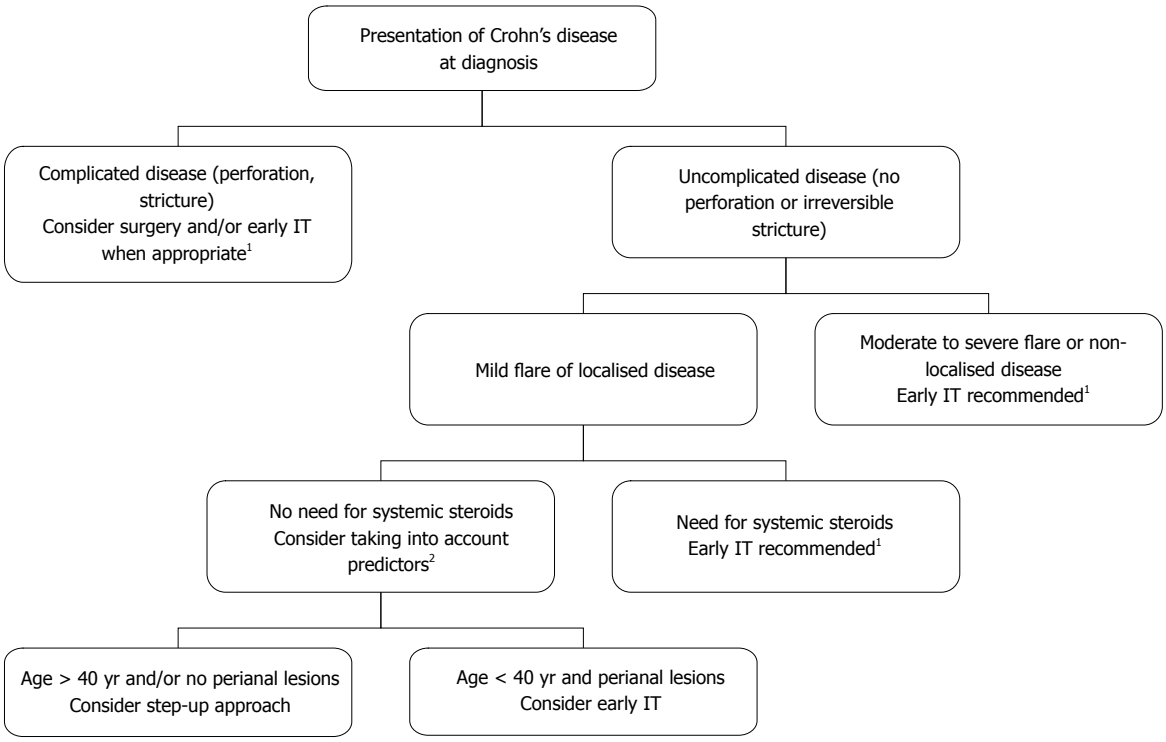

Figure 1 Indication for early immunosuppressive therapy. Thiopurines and/or anti-tumor necrosis factor therapy, according to presentation of Crohn's disease at diagnosis, [1]European Crohn's and Colitis Organisation guidelines and [2]Saint-Antoine predictors (2 or 3 of the following items at diagnosis are predictive for subsequent 5-year unfavourable course: Age < 40 years, need for systemic steroids and presence of perianal lesions). IT: Immunosuppressive therapy.

with systemic steroids is increasingly considered as an indication for concurrent initiation of immunosuppressive therapy[54], taking into account, for instance, the high rate of recurrence within the year after steroid discontinuation[55]. Among the patients treated with systemic steroids from diagnosis, it must be noted that those who are younger than 40 years and/or have perianal lesions at diagnosis have *ipso facto* at least two predictors of unfavourable subsequent disease. Finally, patients with first disease flare not requiring systemic steroids often have localised[28] (< 30 cm) continuous intestinal disease with mild symptoms. In this context of true initial benign disease only, Saint-Antoine predictors may be helpful for the decision: in particular, young patients with perianal lesions could be considered for early immunosuppressive therapy, given the high risk of subsequent unfavourable course. However, if early immunosuppressive therapy is not decided on and if patients go rapidly into clinical remission, subclinical inflammatory lesions can now be monitored using blood C-reactive protein, magnetic resonance imaging and faecal calprotectin evaluations, limiting the risk of subclinical progression of intestinal lesions towards irreversible damage without appropriate change in maintenance treatment.

Clinical predictors at surgery of postoperative recurrence and choice of the postoperative maintenance treatment: After intestinal resection, all previous smokers are strongly encouraged to quit smoking. For guiding the choice of the immediate postoperative treatment, in the absence of a validated predictive index, the members of the ECCO consensus group have selected some of the predictors reported in the literature series[28]. In addition to the two undisputed predictors of early recurrence (keeping smoking and prior resection), penetrating disease behaviour, perianal disease and extensive small bowel resection have been proposed as risk factors. In patients with at least one risk factor, thiopurines are proposed as the drug of choice for preventing early recurrence. However, it is also recommended to perform during the first postoperative year an ileocolonoscopy in order to assess the presence and severity of endoscopic perianastomotic lesions. These endoscopic findings are unanimously considered as the gold standard predictor of subsequent clinical evolution[26,27], so that the definite choice of the postoperative prophylactic treatment should be based on the severity of endoscopic lesions[28]. As a consequence, the choice of immediate therapy based on (insufficiently) validated clinical predictors *vs* tailored treatment initiated according to endoscopic findings has a limited impact since, in all patients, the final choice of the mid-term prophylactic treatment is based on endoscopic findings.

UC

Clinical predictors of UC course

Prediction at diagnosis of unfavourable 5-year or 10-year clinical course: This question has been specifically studied in five population-based studies (Table

1)[2,4,12,56,57]. Young age at diagnosis and female gender were associated in two studies with a trend towards more frequent relapses[12,56]. Active smoking status was associated with reduced number of relapses within 10 years after diagnosis in one cohort only[56], but many other studies on the natural history have established the relationship between sustained non-smoking status and less active disease course[1], with reversion of this impact when resuming smoking[15]. However, of course, patients with UC are not encouraged to smoke. Finally, in the Danish cohort, a high level of systemic clinical symptoms at presentation (fever, weight loss) was associated at the same time with a trend towards infrequent relapses and chronic disease but higher risk of colectomy. Since proctocolectomy is no longer considered as the end of the medical problems in most operated patients, the risk of colectomy is a good marker of overall severity in patients with UC. Extensive colitis at presentation (defined as upper limit of macroscopic lesions proximal to the splenic flexure) has consistently been evidenced as an independent predictor of colectomy within the 10 years after diagnosis.

Serological and genetic predictors of UC course

Serological and genetic predictors have been far less studied in UC than in CD and only a few clinical settings have been investigated. As in CD, the severity of UC does not seem to be affected by family history of IBD[12,58]. In the pre-colectomy situation, high levels of pANCA have been associated with the risk of subsequent chronic pouchitis in UC patients undergoing IPAA[59]. pANCA might also be useful to predict response to anti-TNF therapy since negative status has been independently associated with early response to this treatment[60]. Some genetic factors might also be useful to predict response to treatment in the future. A recent study by a German group suggests that homozygous carriers of IBD risk-increasing *IL23R* variants are more likely to respond to infliximab than are homozygous carriers of IBD risk-decreasing *IL23R* variants[60]. It is also possible that a genetic scoring system taking into account several single nucleotide polymorphisms might be able to identify medically refractory UC patients[61]. As in CD, serological and genetic markers will probably be useful in future composite predicting tools.

Relevance and potential use of predictors in UC

Compared with CD, individual natural history of patients is harder to predict than in CD[1], and the clinical relevance of clinical predictors of 10-year UC severity has not been validated in independent cohorts. However, extensive colitis at diagnosis consistently appears as a predictor of subsequent colectomy. In addition, extensive colitis is strongly associated with the risk of long-term colorectal cancer[62,63], and persisting colonic mucosal inflammation (macroscopic[64] and microscopic[65]) also contribute independently to the risk. Finally, the IBSEN group also demonstrated that obtaining mucosal healing in the short-term in patients with UC is associated with a reduced risk of subsequent colectomy[66]. In this context, it is reasonable to suggest considering a more aggressive therapeutic strategy (for instance, faster step-up approach) in patients with extensive colitis at diagnosis.

In conclusion, at the moment no reliable genetic or serological predictor of IBD course can be used in clinical practice. Saint-Antoine clinical predictors of subsequent unfavourable CD course may be taken into account in the decision about early immunosuppressive therapy in selected patients, and extensive colitis at diagnosis should influence individual therapeutic strategy in UC. However, we are aiming now towards the primary therapeutic goal of obtaining sustained deep remission (including mucosal healing) rather than simple sustained clinical remission. Consequently, we will need to identify predictors at diagnosis of subsequent sustained deep remission. Given the growing research in genetics and pharmacogenomics, we can imagine that sensitive and reliable predictors will be available in the near future.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Impact of environmental and dietary factors on the course of inflammatory bowel disease

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Abstract

Besides their possible effects on the development of inflammatory bowel disease (IBD), some environmental factors can modulate the clinical course of both ulcerative colitis (UC) and Crohn's disease (CD). This review is mainly devoted to describing the current knowledge of the impact of some of these factors on the outcome of IBD, with special emphasis on smoking and diet. Although the impact of smoking on the susceptibility to develop CD and UC is firmly established, its influence on the clinical course of both diseases is still debatable. In CD, active smoking is a risk factor for postoperative recurrence. Beyond this clinical setting, smoking cessation seems to be advantageous in those CD patients who were smokers at disease diagnosis, while smoking resumption may be of benefit in ex-smokers with resistant UC. The role of dietary habits on the development of IBD is far from being well established. Also, food intolerances are very frequent, but usually inconsistent among IBD patients, and therefore no general dietary recommendations can be made in these patients. In general, IBD patients should eat a diet as varied as possible. Regarding the possible therapeutic role of some dietary components in IBD, lessons should be drawn from the investigation of the primary therapeutic effect of enteral nutrition in CD. Low-fat diets seem to be particularly useful. Also, some lipid sources, such as olive oil, medium-chain triglycerides, and perhaps omega-3 fatty acids, might have a therapeutic effect. Fermentable fiber may have a role in preventing relapses in inactive UC.

Key words: Environmental factors; Dietary factors; Non-steroidal anti-inflammatory drugs; Smoking; Infections; Inflammatory bowel disease

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INTRODUCTION

Despite the advances in uncovering genetic risk for Crohn's disease (CD) and ulcerative colitis (UC) over the past decade, the etiopathogenesis of inflammatory bowel diseases (IBDs) cannot be explained only in terms of genetic susceptibility. In fact, a vast number of possible environmental risk factors for the development of IBD have

been investigated, including smoking, dietary factors, psychological stress, use of non-steroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives, appendectomy, breastfeeding, as well as infections and other events related to the so-called "hygiene hypothesis" in childhood[1].

In addition to their putative effects on the development of IBD, some environmental factors can play a role in modulating the clinical course of both UC and CD. This review is mainly devoted to describing the current knowledge of the impact of some of these factors on the clinical outcome of IBD, with special emphasis on smoking and diet. The role of microbial factors (namely, the commensal microflora, and pathogens such as *Mycobacterium avium ssp*. paratuberculosis) in the pathogenesis of IBD will be not discussed.

SMOKING

Tobacco is the best established environmental factor affecting the susceptibility to develop IBD[2] and maybe its clinical course, with opposite effects in CD and UC. However, most of the published studies assessing the impact of smoking on the long-term clinical outcomes of IBD are retrospective, often leading to controversial results.

Role of smoking on the clinical course of CD

Beyond the higher incidence of CD among smokers, several studies suggest that continuing to smoke leads to worse clinical outcomes[3]. The underlying mechanisms of this deleterious effect are not well understood, but it has been reported that tobacco glycoprotein may be responsible for promoting a Th1 cell response[4]. Moreover, tobacco increases production of reactive oxygen species and impaired antioxidant capacity has been shown in smokers[5].

Smoking has been associated with a higher risk of relapse[6,7] and increased need for immunomodulators[8], but the strongest evidence of the deleterious effect of smoking upon the course of CD lies in the beneficial consequences of smoking cessation[9]. Cosnes *et al*[10] demonstrated that patients who stop smoking for at least 6 mo are at a lower risk of relapse in the following 12-18 mo, as compared to non-quitters.

The negative impact of active smoking may not be the same in all CD patients and it has been suggested that it may depend, at least, on gender and disease location. The effect of smoking has been reported to be more marked in women[8,10]. While the natural history of colonic CD seems to be the same in smokers and non-smokers, the rates of relapse[11] and intestinal resection are higher among smokers with ileal disease[6]. In addition, smoking has been associated with a lower prevalence of inflammatory (non-stricturing, non-penetrating) behavior of the disease, thus suggesting that tobacco facilitates progression towards complicated disease[12-14]. Nevertheless, this may only reflect a greater proportion of smokers among patients with ileal involvement[15].

The negative effects of tobacco seem to be dose-dependent, and some studies pointed to an increased risk of surgery and persistent inflammatory activity in those patients smoking over 10 cigarettes/d[12,16]. Conversely, a recent study reported non-detrimental effects of active smoking on CD course, but passive smokers needed immunosuppressants and infliximab more frequently than non-passive smokers[17]. Although seldom assessed, genetic background may also play a role, as suggested by the lack of association between smoking and CD in Jewish patients in Israel[18].

The worse clinical evolution among smokers might also be explained by a lesser response to drug therapies. Despite initial data suggesting a decreased likelihood of response to infliximab for luminal CD in smokers[19,20], larger studies failed to find any association between smoking and infliximab response[21-23]. We assessed the influence of smoking on the response to thiopurines in steroid-dependent IBD and, although no differences between smokers and non-smokers were found, CD responders who continued smoking had a higher rate of relapses during follow-up. Surprisingly, we found that smoking was an independent predictor for the need of thiopurine discontinuation because of side effects, leading to a lower treatment efficacy among CD patients (as compared to UC) when evaluated by intention-to-treat analysis[24].

Postsurgical recurrence is the clinical scenario in which active smoking has better proven to worsen CD prognosis both in the short- and long-term[25]. Smoking has been reported to be an independent risk factor for endoscopic, clinical, and surgical recurrence[26-28]. We have recently reported the results of the first prospective study assessing risk factors for endoscopic recurrence in a series of 152 CD patients undergoing ileo-colic resection[29]. Smoking was independently associated with significant postoperative recurrence as defined by the development of clinical recurrence and/or Rutgeerts grade 3 or 4 of endoscopic recurrence, whereas the only independent protective factor was the use of thiopurines[29]. Of interest, postoperative recurrence has been reported to be much more marked among heavy smokers (> 10 cigarettes/d)[26,29]. In spite of the fact that it has been suggested that the harmful effect of tobacco in CD might be neutralized by the use of immunomodulators[10], this does not seem to be the case in postoperative recurrence where two different studies identified both azathioprine use and active smoking as independent predictors for both endoscopic and surgical recurrence[26,29].

Role of smoking on the clinical course of UC

There is strong evidence pointing to a protective effect of tobacco on the susceptibility to develop UC[2]. Early studies, performed before the widespread use of calcineurinic drugs and infliximab, suggested higher rates of flares, hospitalizations, and even colectomy for UC among non-smokers[30-33], but these findings were not confirmed in most recent studies[34,35]. Conflicting results have been obtained about the effect of tobacco on retrograde progression of distal forms of UC[31,36-38].

Some authors reported a worsening in clinical out-

comes among UC patients who quit smoking[30,39], while improvement of disease activity has been noticed in ex-smokers who returned to smoke[40,41]. A number of trials show the efficacy of nicotine for inducing remission in active UC, although with a high rate of mild side effects[42]. In fact, some authors still propose "mild smoking" as an alternative therapy in patients with resistant UC[43].

DIET

Along with microbiota, dietary products are the most common luminal antigens in the bowel and may influence intestinal inflammation. Possible mechanisms include a direct antigenic effect, alteration of gene expression, modulation of inflammatory mediators (e.g., eicosanoids), changes in the composition of the enteric flora, and effects on gut permeability. Thus, the role of dietary habits on the development of IBD has been extensively investigated in case-control retrospective studies subject to different biases[44,45]. In a recent systematic review of these studies, high intakes of total fat, omega-6 fatty acids and meat were associated with increased risk of developing IBD, while high vegetable and fruit intake decreased the risk for these diseases[45]. A recent case-control study suggests that increased intake of refined sugars may facilitate the development of CD and UC[46]. Prospective studies are necessary to confirm the role of dietary factors on the development of IBD.

Role of diet on the clinical course of IBD

For decades, physicians based dietary counseling for IBD patients on restrictive criteria. This was because the so-called "bowel rest" was considered as a sine qua non to induce disease remission. However, controlled trials clearly demonstrated that drug-induced IBD remission was not influenced by the type of nutritional support (i.e., enteral, parenteral or oral conventional foods)[47-49]. Thus, the concept of "bowel rest" has been abandoned, and IBD patients are now advised to eat a diet as unrestricted as possible.

Food intolerance: Does it have a role in dietary management of IBD? IBD patients often complain of food intolerance. In a prospective study, 65% out of 130 patients who completed a food questionnaire reported to be intolerant to some food item, as compared to only 14% out of 70 healthy controls ($P < 0.0001$)[50]. A more recent study in 187 UC patients confirms these findings: 50% of patients avoided some foodstuff (mainly dairy foods, fruits and vegetables)[51]. However, 22% of patients ate supplemental amounts of these food items because they had the perception that these improved their symptoms[51].

Despite its high prevalence, food intolerance is quite inconsistent in IBD patients. Pearson *et al*[52] sequentially introduced single conventional foods in 28 CD patients who had gone into remission with an elemental diet. Twenty patients reported intolerance to some of these foods, but seven of them were tolerant to it after a re-challenge. Of interest, one patient who was also intolerant to this rechallenge, could tolerate the "offending" food after a second blinded rechallenge, and someone even had opposite responses to two blinded rechallenges with the same food item[52].

These data well illustrate how difficult it is to prove food intolerances in IBD patients. From this perspective, avoiding every food that causes patient's upset is an unwise strategy. In a large series of patients with inactive UC, dietary changes based on the patient's self-perceptions did not have any influence on the relapse rate[51]. Therefore, bearing in mind the fact that protein-energy malnutrition and other nutritional deficiencies are frequent in IBD, patients with UC or CD should be advised to avoid only those food items which repeatedly and systematically worsen their symptoms. In this setting, two groups of foods often raise concerns both among patients and doctors: dairy foods and dietary fiber.

None of the milk components has been proven to play a role in promoting bowel inflammation, causing the disease or triggering a flare. In contrast, it is well known that dairy foods are the main dietary source of calcium, which is necessary to prevent metabolic bone disease in these patients. However, it is also true that a significant proportion of healthy people (mainly in the Mediterranean basin) have lactase deficiency. Unabsorbed lactose reaching the colon may cause diarrhea and/or bloating in a dose-dependent manner. This phenomenon, which does not depend on the fact of suffering from IBD, may occur in lactase-deficient patients with these diseases, thus worsening their symptoms. Studies performed in our laboratory suggest that the prevalence of lactose malabsorption (as assessed by hydrogen breath test) is not higher in IBD patients than in healthy controls[53]. Therefore, IBD patients should not limit their milk intake during flares unless it clearly worsens diarrhea. Moreover, even in these cases, dairy foods with lower lactose contents (i.e., yogurt) may be well tolerated.

Prescribing a low-residue diet - that is, devoid of insoluble fiber - may be advisable during acute flares of IBD, particularly in patients with stricturing CD or severe UC attacks. Soluble fiber generates much less residue than insoluble fiber, and is fermented by colonic microflora yielding several products such as short-chain fatty acids (SCFA) - mainly butyrate - than can be of benefit in IBD. Butyrate is the preferred fuel for colonic epithelial cells. Decreased fecal levels of SCFA have been reported in patients with UC in relation to the severity of inflammation[54], and impaired beta-oxidation of butyrate could be demonstrated in patients with active and even inactive UC[55,56]. Experimental work suggests that butyrate is able to down-regulate the production of proinflammatory cytokines, and also nuclear factor kappa B (NF-κB) activation[57].

Soluble fiber may be particularly useful in inactive UC. In a randomized controlled trial, *Plantago ovata* husks (a source of slowly fermentable soluble fiber) were as

effective as mesalazine for preventing disease relapse in patients with quiescent UC[58]. In active UC, however, the use of soluble fiber might be potentially detrimental. The presence of intraluminal blood (and, hence, oxygen), and a lower intraluminal pH during active disease may favor the growth of lactic acid-producing bacteria (*Lactobacilli* and *Streptococci*). Lactic acid directly damages the bowel mucosa. Indeed, increased levels of fecal lactic acid have been reported in patients with active UC[59].

The usefulness of "exclusion diets" in CD has been supported by several authors due to their potential capacity to prevent clinical relapses and spare steroids. To date, only one prospective randomized controlled trial assessing the role of exclusion diet in preventing relapse in inactive CD has been published[60]. Seventy-eight patients, who had gone into remission with an elemental diet, were randomized to receive an exclusion diet (i.e., sequential introduction of foods, with exclusion of those that elicited symptoms) or prednisolone (40 mg/d, with tapering dose until discontinuation by week 12) (control group)[60]. Treatment of a control group is hard to justify, since it is well-known that steroids are not useful as maintenance therapy in CD. Anyway, the two-year cumulative probability of relapse was lower in the group treated with the exclusion diet than in the control group (62% *vs* 79%, $P = 0.048$)[60]. However, 62% is a high relapse rate, suggesting that exclusion diets benefit only a minority of CD patients.

Food components as primary treatment for CD: In the last three decades, the possibility that enteral nutrition could be used as primary treatment (i.e., able, *per se*, to induce remission) in active CD has been a matter of debate.

To date, four meta-analyses of the trials comparing enteral nutrition *vs* corticosteroids in active CD have been published[61-64]. All of them agree that steroids are better than enteral nutrition in inducing remission but they also indicate that, as a whole, enteral nutrition is able to induce remission in about 50%-60% of patients, a remission rate substantially higher than that obtained with placebo in active CD, which barely achieves 30%. This suggests that enteral nutrition (or, at least, some enteral formulas) would have a primary therapeutic effect in active CD (or, at least, in some subsets of patients). The primary therapeutic effect of enteral nutrition in CD is particularly relevant for children, as confirmed by two meta-analyses of pediatric trials which conclude that enteral nutrition is as effective as steroids in inducing remission in children[65,66]. In addition to its role in active CD, enteral nutrition is suggested to be useful for preventing relapse both in children[67] and adults[68]. Recent data suggest that it could also have a role in preventing postoperative recurrence[69].

The mechanisms whereby enteral nutrition exerts its primary therapeutic effect in CD remain obscure. The hypothesis that elemental (i.e., amino acid-based) diets would be particularly useful by virtue of their low antigenicity was challenged by the results of meta-analyses of randomized trials comparing elemental *vs* non-elemental (i.e., peptide- or whole protein-based) diets, which showed that both types of diets were equally effective in inducing remission[61,64].

To date, the amount and/or the type of dietary fat are major candidates for the therapeutic effect of enteral nutrition in CD. Recent meta-analysis suggests very low fat (i.e., less than 3 g/1000 kcal) diets could be particularly effective[64]. Early studies pointed out that olive oil-based diets were better that diets based on seed oils (corn, safflower, sunflower, soybean), suggesting that oleic acid would be better than linoleic acid in reducing inflammation[70]. Experimental data also support this view[71,72]. However, this hypothesis could not be confirmed in a trial comparing linoleic acid- and oleic acid-based diets, where the latter performed particularly badly[73]. As the oleic acid source in this trial was not olive oil but synthetic triolein, it cannot be ruled out that other components of olive oil (e.g., antioxidants) could exert anti-inflammatory actions in these patients.

Although coconut oil-derived medium-chain triglycerides (MCT) are traditionally considered as a mere easy-to-oxidize energy source, recent data support the idea that they can also exhibit immunomodulatory properties. In fact, there is growing experimental evidence that MCT are able to improve bowel damage both in spontaneous and induced animal models of intestinal inflammation[74-77]. There are also some clinical data suggesting that replacing part of dietary fat with MCT would contribute to the primary therapeutic effect of enteral nutrition in CD[78-80].

Surprisingly, fish oil-derived omega-3 fatty acids - the paradigm of anti-inflammatory lipids - have been scarcely assessed in the setting of enteral nutrition formulas for CD. Several randomized trials have been published, however, on the role of fish oil supplements as therapy for both active and inactive CD and UC, which have been systematically reviewed[81-83]. Overall, available data do not allow supporting the use of omega-3 fatty acid supplementation for the treatment of both active and inactive IBD. Negative results are quite consistent in trials assessing the use of omega-3 fatty acids to maintain disease remission, particularly UC, and to a lesser extent CD. Trials on their use in active disease do not allow us to draw firm conclusions, mainly because of the heterogeneity of their design (UC) or their small number (CD). In most trials, the appropriateness of the selected placebo is questionable[83].

NSAIDs

Since it is known that NSAIDs can induce gastrointestinal mucosal inflammation, it has been suggested that they might trigger disease exacerbation in IBD patients. Several potential mechanisms for this phenomenon have been proposed such as cycloxygenase (COX) inhibition, leukotriene shunting or inhibition of NF-κB activity, although none of them has been firmly demonstrated[84].

Most (but not all) retrospective, uncontrolled or cross-sectional studies evaluating the impact of NSAID use on IBD relapse agree on the potential deleterious effect of these drugs on quiescent IBD[84,85]. In the only prospective controlled study assessing disease relapse with the use of different NSAIDs as compared to acetaminophen in IBD patients without arthritic complaints, a significantly increased risk of relapse with NSAIDs was reported[86]. Interestingly, patients who tolerated NSAIDs for a week did not seem to be at risk for relapse, suggesting that drug-induced IBD flares occur early after starting NSAID use and only in a subset of susceptible patients. It is also still debated whether selective COX-2 inhibitors are safer than conventional NSAIDs for patients with IBD. The only prospective, randomized, double-blind, controlled trial performed to date showed no increase in UC flares as compared to placebo[87], but most authors conclude that further randomized, double-blind trials are needed to address this issue[84,85].

INTESTINAL INFECTIONS

Intestinal infections by enteropathogens have been associated with both IBD onset and IBD relapses, and stool microbiological studies are usually advised in patients with IBD flares. Several prospective and retrospective studies show that intestinal infections assessed by stool cultures occur in less than 10% of IBD flares, mainly in those patients with extensive colonic involvement[88-93]. However, the clinical relevance of such infections on IBD course has not been appropriately assessed, and no study has specifically addressed the effect of adding antibiotic therapy in patients with active IBD and a positive stool culture. Baliellas *et al*[89] reported that only half of the patients with active IBD and positive stool cultures achieved symptomatic remission after antibiotic therapy alone, despite stool cultures becoming negative in all of them.

Clostridium difficile infection (CDI) has become in recent years a worldwide epidemic phenomenon also affecting IBD patients. In the last two decades, the prevalence of CDI increased two-fold in UC and almost three-fold in CD[94]. As for enteropathogen intestinal infections, IBD patients with colonic involvement seem to be those at higher risk for CDI[95-97]. In addition to the risk factors for CDI in the general population, increasing age and steroid use seem to be particularly relevant in IBD patients, with no conclusive data about the role of other immunosuppressants[98]. Several studies have reported that CDI worsens IBD outcome, with higher rates of surgical procedures, longer hospital stay, and higher mortality, as compared to patients admitted to the hospital with IBD or CDI alone[94,95,97,99].

Finally, many studies have been published addressing the role of intestinal cytomegalovirus (CMV) infection in IBD, mainly in UC[100]. Two prospective studies demonstrated that this infection was a reactivation of CMV carriers that occurs almost exclusively in active, steroid-refractory UC patients[101,102]. However, the small number of patients included in both studies does not allow ascertaining whether CMV reactivation is the cause of refractoriness or the consequence of steroid use, and also whether it worsens UC outcome or if it is only an innocent bystander.

OTHER ENVIRONMENTAL FACTORS

Breastfeeding

Breastfeeding is a protective factor against several immunologically-based diseases. In fact, breast milk is relevant for acquiring tolerance against bacterial microflora and dietary antigens. Most studies investigating the role of breastfeeding on the development of either UC or CD have shown a protective effect, as concluded in a meta-analysis of 14 case-control studies published in 2004[103]. Subsequent studies confirmed these results (especially for those infants breastfed for more than six months)[46], while others suggest that breastfeeding could promote CD in childhood, rather than protecting from its development[104].

Obesity

IBD, particularly CD, has been traditionally associated with protein-energy malnutrition and other nutritional deficiencies. However, in recent years the prevalence of obesity among IBD patients has been increasing[105] in parallel with the obesity epidemics in the general population of developed countries. Case-control studies suggest that obese CD patients are more prone to perineal disease[106] and early surgical treatment[107].

Vaccinations

The role of vaccinations - mainly attenuated measles-containing vaccines - in the development of IBD is a matter of debate, with studies reporting a positive[108], negative[109] or no association[110,111] with IBD. A recent case-control study reported that vaccinations against pertussis and polio increase the odds of suffering IBD[46]. The exact role, if any, of vaccinations with regard to IBD is far from being elucidated.

Oral contraceptive pills

A meta-analysis conducted in 2008 showed that the use of oral contraceptives was associated with an increased risk of both CD and UC[112]. The risk increased with the time of exposure and reversed after pill discontinuation[112]. The effect of oral contraceptives on the risk of IBD appears to be related to estrogens. Estrogen acts as an immune enhancer and may increase the production of tumor necrosis factor by macrophages. Also, estrogen may induce microthrombotic phenomena in the bowel due to its thrombogenic potential.

Appendectomy

Appendectomy is associated with a lower risk of suffering from UC, particularly in children who are operated

before the age of 10, as shown in a meta-analysis of 17 case-control studies[113]. Investigations on the relationship between appendectomy and CD are less conclusive, in spite of the fact that a recent meta-analysis showed an increased risk of CD in appendectomized subjects[114]. However, this association was particularly strong for those appendectomies performed within one year before CD diagnosis, and almost null for those performed five years before CD[114], suggesting that this is a circumstantial rather than causative relationship.

Psychological stress

Psychological stress has been hypothesized to play a role both in the pathogenesis of IBD and as a triggering factor for disease relapse as well. However, retrospective observational studies have yielded conflicting results[115,116].

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Impact of medical therapies on inflammatory bowel disease complication rate

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Abstract

Crohn's disease and ulcerative colitis are progressive diseases associated with a high risk of complications over time including strictures, fistulae, perianal complications, surgery, and colorectal cancer. Changing the natural history and avoiding evolution to a disabling disease should be the main goal of treatment. In recent studies, mucosal healing has been associated with longer-term remission and fewer complications. Conventional therapies with immunosuppressive drugs are able to induce mucosal healing in a minority of cases but their impact on disease progression appears modest. Higher rates of mucosal healing can be achieved with anti-tumor necrosis factor therapies that reduce the risk of relapse, surgery and hospitalization, and are associated with perianal fistulae closure. These drugs might be able to change the natural history of the disease mainly when introduced early in the course of the disease. Treatment strategy in inflammatory bowel diseases should thus be tailored according to the risk that each patient could develop disabling disease.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Therapy; Surgery; Complications

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INTRODUCTION

The natural history of Crohn's disease (CD) is the progression to chronic complications including strictures, penetrating fistulae[1,2] or complex perianal disease[3], leading to the need for surgery and hospitalization[4]. This leads to the concept of cumulative tissue damage for which a quantitative score is currently under development[5]. Coloproctectomy due to chronic refractory disease or acute severe colitis is a major complication of ulcerative colitis (UC) and develops in 20%-25% of patients after 25 years. An increased risk of colorectal cancer (CRC) in long-standing colitis is a second major complication in UC[6], but also in Crohn's colitis, with a relative risk of 2.5 compared to the general population. The aim of medical therapies was the improvement of inflammatory bowel disease (IBD) symptoms 20 years ago, however, the current objective is to achieve deep remission, including cessation of corticosteroids, and mucosal healing. Therefore, treatments should modify the course of the disease by avoiding disabling disease and irreversible tissue damage. This review focuses on the impact of treatment on the natural history of IBD.

MUCOSAL HEALING

Mucosal healing (MH) has become a major goal of treatment of IBD because it has been correlated with fewer complications[7,8], fewer relapses after surgery[9], and drug

withdrawal[10]. There is no validated definition of MH in IBD. Mucosal healing is usually assessed by endoscopy in CD and UC and defined as the absence of ulcers[10]. Although poorly studied, MH is achievable with thiopurine analogs in active CD. In earlier uncontrolled studies, among azathioprine (AZA) clinical responders, 74% achieved MH after a mean of 2 years[11,12]. However, in a more recent controlled trial (SONIC) studying infliximab (IFX), AZA, or combination therapy for immunosuppressive-naive CD patients[13], only 16% of CD patients in the AZA arm achieved mucosal healing at week 26. Few data are available about the efficacy of methotrexate (MTX) in inducing MH. A preliminary study[14] of 11 CD patients treated with MTX 25 mg weekly intramuscular injection showed MH and histological healing in five and four patients, respectively, after 12 wk. No MH was observed in UC, although 5/7 patients had a clinical response with histological improvement. A recent prospective study showed MH in only 11% of CD patients in clinical remission on MTX, compared to 50% on AZA and 60% on IFX[15]. A possible bias in this study may have been the small size of the groups, the more refractory disease, and the shorter treatment duration in the MTX group. Anti-tumor necrosis factor (TNF) treatments have changed the management of IBD since the late 1990s. A subanalysis of a Crohn's disease clinical study evaluating infliximab in a new long-term treatment regimen (ACCENT1) trial demonstrated that MH on IFX was associated with fewer relapses[16]. A retrospective single center study has shown that, among IFX responders, 68% had MH (45% complete MH) and MH was associated with fewer relapses (64% *vs* 40%)[17]. In the step-up top-down study, 71% of patients with MH at 2 years were still in remission 2 years later, compared to patients who had endoscopic signs of activity[18]. At week 26 of the SONIC trial, IFX was more effective to induce MH than AZA, (16.5%), either in mono- (30.1%) or combination therapy with AZA (39.5%). In the prospective ACT1/ACT2 trials studying IFX for induction and maintenance therapy in UC, IFX efficacy in inducing MH was also demonstrated with 62%/60% of MH at week 8 compared to 32%/30% in the placebo group. Adalimumab (ADA) was also more effective than placebo in inducing and maintaining clinical remission in patients with moderate-to-severe UC[19], and MH was achieved more frequently in the ADA arm compared to placebo (25% *vs* 15% at week 52).

In CD, mucosal healing has also been consistently described as more frequently achieved when an anti-TNF was started earlier in the disease course[20].

SURGERY AND HOSPITALIZATION

CD is a chronic condition that leads to tissue damage and complications requiring surgery in 70%-80% of patients at 20 years[4,8]. In UC, the cumulative probability of colectomy after 25 years varies from 20% to 30%[21,22]. In 2005, Cosnes *et al*[23] demonstrated that immunosuppressive drugs (AZA and MTX) were introduced more frequently and earlier in the course of the CD over the past 25 years but the percentage of patients requiring intestinal surgery each year remained stable. These results should be interpreted with caution because < 10% of the patients included in this study received AZA before surgery. Recent contradictory data have demonstrated that increased immunosuppressant prescriptions, from 11% to 45% over 25 years, have decreased the rate of intestinal resection from 59% to 25% 5 years after diagnosis[24]. Early introduction of thiopurine was a protective factor. French results recently have demonstrated that AZA is associated with less surgery in patients newly diagnosed with CD but the benefit was modest compared to IFX[25]. In UC, Ardizzone *et al*[26] have demonstrated higher rates of clinical response in patients treated with AZA compared to 5-aminosalicylic acid, but the colectomy rate was similar in both groups (8%). The ACCENT 1 and 2 trials have reported a decreased risk of surgery in patients on IFX scheduled therapy at week 54 (3% *vs* 7% with IFX episodic therapy)[27,28]. Schnitzler *et al*[17] have demonstrated less intra-abdominal surgery (14%) and hospitalization (42%) for active CD in patients achieving MH on scheduled IFX compared to those who had endoscopically active disease (38% and 59% respectively). Lower colectomy rates in IBD were also associated with MH in a retrospective Norwegian population-based study[29]. Recently, IFX given for at least 16 mo was reported as a protective factor against surgery in active CD[25]. Jones *et al*[30] have reported a stable rate of surgery in CD from 1993 to 2004, but these data should be interpreted with caution because they concern a period when IFX was mainly prescribed as episodic therapy, which is clearly a suboptimal strategy and does not represent the current practice. In the Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance (CHARM) trial, CD patients treated with scheduled ADA had less hospitalization at 3 mo (1.6%) and 12 mo (5.9%) compared to placebo (7.3% and 13.9% respectively)[31]. Surgery at 1 year also decreased from 3.8% in the placebo group to 0.6% in the ADA groups. These results were confirmed at 2 years follow-up[32]. A sub-analysis of the ACT1 and ACT2 trials demonstrated a 10% cumulative incidence of colectomy in UC through 54 wk in the IFX group compared to 17% in the placebo group. Less UC-related hospitalization was reported in the IFX group[33]. Moreover, the degree of MH after 8 wk IFX was correlated with less colectomy[34].

PERIANAL COMPLICATIONS OF CD

Perianal fistulae occur in 21%-26% of CD patients after 20 years[3]. Anti-TNF antibodies have dramatically improved the ability to heal fistula with medical therapy. After 54 wk of scheduled IFX treatment for active CD fistulae, partial response was observed in 46% of patients *vs* 23% in the placebo group and 36% had a complete response compared to 19% with placebo[28]. After primary drainage, high rates of clinical response (85%)

and remission (74%) at week 14 were also reported in patients with severe active perianal CD treated with three infusions of IFX followed by MTX as maintenance therapy (25 mg weekly intramuscular or subcutaneous). Fifty percent of patients were still in remission from their perianal disease at 1 year, but this strategy failed to achieve a prolonged remission of luminal disease in the majority of patients[35]. No other prospective studies have investigated the efficacy of MTX in perianal CD. In the CHARM trial that studied the long-term efficacy of ADA in CD, 33% of active fistulae achieved complete healing on ADA after 56 wk compared to 13% in the placebo group[31]. This effect was globally maintained over 3 years follow-up[32]. Fistula healing in a substantial proportion of patients under ADA was also confirmed in a large European open label trial mimicking routine practice[36]. In this trial, full fistula closure was achieved in 26% of patients after 20 wk. The impact of thiopurine on fistula closure was poorly studied. A meta-analysis showed a complete closure or an improvement of the fistulae in 54% of the patients compared to 21% in the placebo group. However, these results should be interpreted with caution because fistula closure was not the primary endpoint of this study[37].

CANCER

Chronic colitis predisposes to CRC over time, with cumulative estimated incidence rates of 2%, 8% and 18% at 10, 20 and 30 years of evolution, respectively[6]. This risk was however reported as lower in more recent cohorts[38], with a relative risk around 2.0 over disease course. The risk of CRC in CD has also been reported[39]. Risk factors for CRC in chronic colitis are extensive location, long duration of the disease, familial history of CRC, and associated primary sclerosing cholangitis[40]. Few studies have addressed the severity of colonic inflammation over time as an independent risk factor for progression to neoplasia. Rutter *et al*[41,42] have demonstrated a highly significant correlation between colonic inflammation scores and the risk of CRC in UC. Only association with histological inflammation was significant in the multivariable analysis [odds ratio (OR): 4.7]. In the case of normal colonoscopy, the 5-year risk of CRC was the same as that of the matched general population (OR: 0.38). Gupta *et al*[43] also have demonstrated that the severity of microscopic inflammation is an independent risk factor for dysplasia in patients with longstanding UC. Such data are not yet available in CD. Due to the ability of medical treatment to maintain tissue healing in IBD, we can speculate on the potential impact of these treatments on the risk of cancer. Mesalazine is effective at maintaining clinical remission in UC and remains the main drug in this disease. In retrospective studies and meta-analyses, a significant decrease in CRC in UC has been described with mesalazine. More intriguingly, this has also been suggested for ileal cancer in CD[44]. More recently, in the Cesame cohort, a potential decrease in CRC was also suggested in extensive longstanding UC treated with purine analogs[45]. No data are available yet with anti-TNF agents.

CONCLUSION

In conclusion, anti-TNFs and to a lesser extent immunosuppressants, can induce MH, which is associated with long-term clinical remission, closure of perianal fistulae, less hospitalization and surgery, suggesting an impact of these medications on the natural history of the disease. The benefit might be higher if MH is achieved earlier in the course of the disease. Histological remission is also associated with a reduced risk of CRC in UC. Although immunosuppressive treatments with thiopurine or MTX are able to induce MH, their benefit on the complications of IBD appears more modest. Many questions remain open, including the degree of MH achievement (complete *vs* partial) required to improve the prognosis, when and how often in the course of the disease should this healing be assessed, and how to adapt the treatment according to MH. Prospective randomized clinical trials are ongoing to answer these questions. Furthermore, the validation and the further use of a tissue damage score in CD (Lemann score) will be an important step to assess adequately the ability of treatment strategies to change natural history.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Surgery for Crohn's disease in the era of biologicals: A reduced need or delayed verdict?

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Abstract

Crohn's disease (CD) is a chronic inflammatory bowel disease that can affect the entire gastrointestinal tract. Ultimately, up to 70% of all patients will need surgery, despite optimized medical therapy. Moreover, about half of the patients will need redo-surgery because of disease recurrence. The introduction of anti-tumor necrosis factor (TNF) drugs (Infliximab in 1998) revolutionized the treatment of CD. Different randomized trials assessed the efficacy of anti-TNF treatment not only to induce, but also to maintain, steroid-free remission. Furthermore, these agents can rapidly lead to mucosal healing. This aspect is important, as it is a major predictor for long-term disease control. Subgroup analyses of responding patients seemed to suggest a reduction in the need for surgery at median-term follow up (1-3 years). However if one looks at population surveys, one does not observe any decline in the need for surgery since the introduction of Infliximab in 1998. The short follow-up term and the exclusion of patients with imminent surgical need in the randomized trials could bias the results. Only 60% of patients respond to induction of anti-TNF therapy, moreover, some patients will actually develop resistance to biologicals. Many patients are diagnosed when stenosing disease has already occurred, obviating the need for biological therapy. In a further attempt to change the actual course of the disease, top down strategies have been progressively implemented. Whether this will indeed obviate surgery for a substantial group of patients remains unclear. For the time being, surgery will still play a pivotal role in the treatment of CD.

Key words: Crohn's disease; Surgery; Biological agents; Anti-tumor necrosis factor drugs; Remission

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder which can affect the complete gastrointestinal tract. Only a minority of patients (10%-15%) will experience a prolonged relapse-free interval after initial diagnosis; most patients develop a mild chronic disease pattern[1]. This relapsing inflammation results in progressive bowel occlusion and/or fistula and abscess formation. A large majority of patients (70%-80%) will require surgical treatment within a time frame of 10 years[2,3]. The type of surgery is dictated by the anatomic location and/or the related complication(s). Depending on the localization of the disease, CD tends to have a different clinical

phenotype. Indeed, ileocolonic and small bowel involvement is more prone to develop occlusive disease than colonic affection[2,4]. Thus, small bowel or ileocolic distribution will increase the rate of surgery compared to Crohn's colitis. Intractable inflammation is a rather seldom indication for surgery. Penetrating anal disease often leads to surgery in order to control sepsis and drain fistulas. Unfortunately, surgery in CD is not curative and the majority of patients will have early endoscopic relapse, despite clinical remission[5]. Over time, symptomatic recurrence demands medical treatment, and up to 40% of patients will eventually need secondary surgery[2]. This explains the tendency to avoid 'too early' surgery. If surgery is needed, the focus should be on bowel sparing and minimally invasive surgical techniques.

Progressive understanding of the pathogenesis of CD resulted in significant changes and improvements in its medical treatment. The use of immunomodulators (such as azathioprine and methotrexate) has not decreased the need for surgery, nor has it decreased hospitalization rates either[2,6]. The introduction of anti-tumor necrosis factor (TNF) treatment in 1998 revolutionized the treatment paradigms. TNF antagonists proved to induce a rapid clinical remission in about 60% of the cases[7,8]. In randomized controlled trials, anti-TNF therapy seemed to maintain remission in contrast to steroid regimens[9-12]. Moreover, mucosal healing has even been obtained in a subset of patients, which could support a sustained clinical remission[13-15]. Therefore, one could expect that, in the long run, fewer patients would need to undergo major abdominal surgery. This paper reflects on some aspects of the impact of anti-TNF treatment on the rates of surgery in CD patients.

NEED FOR SURGERY IN THE MARGIN OF LARGE RANDOMIZED TRIALS

Several randomized controlled trials have analyzed the maintenance of clinical remission in CD comparing patients who received anti-TNF agents or placebo[9-12]. Besides an initial response rate of about 60%, a majority of patients will show sustained remission with anti-TNF therapy. Steroid discontinuation was also significantly better in the treatment groups. Moreover, an endoscopic substudy of a Crohn's disease clinical study evaluating infliximab in a new long-term treatment regimen demonstrated that about 50% of patients with a clinical response will also have mucosal healing[14]. Considering that control of inflammation and induction of mucosal healing is predictive for long-term disease activity and bowel preservation, one could expect an effect of anti-TNF treatment on the rate of surgery[13]. Feagan *et al*[16] evaluated the influence of maintenance adalimumab therapy on the rate of hospital admissions and surgery in a post-hoc analysis of the Crohn's Trial of the Fully Human Antibody Adalimumab for Remission Maintenance trial. The authors came to the conclusion that adalimumab maintenance therapy significantly reduced hospitalizations and surgery for CD amongst the enrolled patients. Mucosal healing seems a promising surrogate marker of deep and prolonged clinical remission. This alteration in disease course should lead to a reduced need for surgery. More predictors are needed, not only to select those patients who will develop an aggressive and complicated disease pattern to enable early installment of immunosuppressive therapy, but also to select patients for "early" surgery to obtain a prolonged clinical remission.

One year after primary surgery, as many as 72% of the patients had already developed endoscopic recurrence, mainly at the anastomosis. Clinical manifestations of the disease are, however, often absent in this early postoperative stage[17]. About one half of patients will need redo-surgery over a 20-year period[2]. Although a high recurrence-rate is observed after surgery, there is no consensus about the postoperative therapy regimen. Considering the high amount of endoscopic recurrences, one could wonder if prophylactic medical therapy after surgery can play a role. Studies have been conducted to find the best prophylactic regimen. Aminosalicylates regimens seem to have modest effects on the postoperative recurrence rate. It is therefore not recommended to use them in a postoperative setting[18,19]. Nitromidazole and ornidazole have demonstrated a significant drop in recurrence, but at the expense of important side effects, making these therapies not longer suitable for prophylactic use. Budenoside has no long term effect, but is indicated to suppress acute relapse. Azathioprine and 6-Mercaptopurine have already been used in randomized controlled trials to assess the effect on rate of recurrence after surgery. Because there was a high drop-out rate during the follow-up period, no convincing results have been found in these series[20,21]. The advent of anti-TNF agents and their demonstrated effect on mucosal healing in the preoperative setting has given hope to care providers that relapses can be avoided when administered postoperatively. Regarding this important clinical question, two randomized controlled trials have been published so far. Twenty-four patients were randomized after ileocaecal resection to receive infliximab or a placebo for one year[22]. The endoscopic and histologic recurrence rate after one year was significantly lower in the infliximab group. There was no significant difference in clinical recurrence rate, though more patients showed relapse after one year in the placebo group. Another study randomized 26 postoperative patients with proven endoscopic recurrence six months after receiving mesalamine in three different groups: one received infliximab , another azathioprine and the last group continued mesalamine[23]. Control of endoscopic inflammation was improved in the infliximab group compared to the azathioprine and mesalamine group, demonstrating the clear suppressive effect of infliximab. In these two small trials, the positive impact of infliximab in avoiding postoperative recurrence has been demonstrated. These conclusions have to be interpreted with the greatest caution considering the small sample sizes and the short follow-up periods. No conclusion can be made about the usefulness of infliximab to

prevent recurrences. Large prospective randomized trials with a long follow-up have to be designed to assess the benefit of anti-TNF agents on postoperative recurrence. Moreover, one could wonder if it is reasonable to give prophylactic treatment after resection, considering the high costs and the number of patients who will be treated that would not develop recurrence. It is more likely to stratify the risk factors of every patient to assess the need of postoperative medical treatment. One of the most powerful methods for assessing patients is performing a colonoscopy six to twelve mo after resection. Rutgeerts *et al*[5] demonstrated the predictive value of endoscopic recurrence. Indeed, patients with severe endoscopic recurrence within one year after surgery are at greater risk of developing clinical recurrence.

Approximately one third of all CD patients will develop perianal disease, including skin tags, ulcers, low and high fistulas, rectovaginal fistulas, perianal abscesses, anorectal strictures and cancer[24]. Complex perianal fistulas are challenging to treat and can lead to destruction of the anal sphincters with intractable incontinence as a result. Twenty-five percent of patients with anal CD will eventually need a proctectomy[25]. The classical medication used for CD, like antibiotics and immunomodulators, have not demonstrated any beneficial effect in the treatment of fistulizing CD[26-28]. In contrast, infliximab maintenance therapy seems to reach superior durable and complete fistula closure, even in patients not responding to other medical treatments[29,30]. This seems to have an impact on the surgery rate and hospital stay[31]. There is, however, some concern about treatment with infliximab inducing healing of the external opening and suppressing the inflammatory reaction around the fistula tract without eradicating the tract[32]. Magnetic resonance imaging of patients in clinical remission after infliximab treatment still showed inflammation and subsisting fistula tracts. Some are concerned about the possibility of fistula recurrence after withdrawal of treatment. More extensive investigation will be needed to test this hypothesis.

NEED FOR A WIDE SURGERY POPULATION (THE REAL WORLD)

In view of the aforementioned randomized controlled trials, it may be possible to change the course of the disease in patients treated with biologicals, perhaps leading to a decreasing need for resectional surgery. Other large population based series, however, are less convincing. Lazarev *et al*[33] showed that, despite the increasing use of infliximab, the rate of small bowel resection has remained unchanged over the years in a large referral centre in Pittsburgh. Moreover, the relative frequency of stricturizing and penetrating disease did not change over time. Bewtra *et al*[34] analyzed hospitalization and surgery trends for inflammatory bowel disease from 1990 to 2003. They observed a steady rate in the number of surgical interventions for CD with a significant increase in hospitalization rate, despite the introduction of infliximab in 1998. Jones *et al*[35] concluded in their series that surgery for penetrating small bowel disease increased with 60% from 1993 to 2004 despite the increasing use of infliximab.

In contrast, two population-based series reported a significant decrease in hospitalization and surgery rate[36,37]. In a series from Wales, stoma formation and the long-term need for steroids are likely to have been influenced by the use of infliximab, but only 16% of patients had been prescribed anti-TNF agents in this series. Moreover, 614 consecutive patients responding to induction therapy with infliximab were observed to evaluate the long term clinical benefit of this anti-TNF agent[38]. Two thirds of these patients seemed to have a sustained benefit of this therapy regimen. There seemed to be a decreased rate of surgery for patients responding to medication. Loss of response was inadvertently associated with an increased risk of surgery. This study demonstrated that infliximab could have an impact on disease course in responding patients. However, this group was not compared to patients not receiving anti-TNF agents. More recently it has been shown that the use of infliximab, and to a lesser extent of azathioprine, seems to be associated with a decreased risk of surgery[39]. Interestingly, in this retrospective cohort study including 296 patients with CD between 2000 and 2008, the median follow-up was 57 mo, which was much longer than in the randomized controlled trials.

DISCUSSION

The introduction of anti-TNF agents significantly changed clinical practice and treatment algorithms. Optimized medical treatment should not only achieve symptomatic relief and clinical remission, but also aim to reduce the need for hospitalization and surgery. The use of these agents evolved over time and is mainly based on evidence from different large clinical trials on the efficacy for induction and maintenance treatment. The achievement of mucosal healing in a subgroup of patients raised the expectation that anti-TNF treatment could lead to a decrease in disease-related complications and the ultimate need for surgery. Biological therapies have shown to alter the natural history of psoriatic arthritis and ankylosing spondylitis[40]. Indeed, anti-TNF therapy has been shown to reduce the need for surgery in different randomized clinical trials. Medium follow-ups of those trials have been rather limited, and the question remains whether the natural history of CD can be changed. Population-wide studies during the anti-TNF era have not yet demonstrated a decrease in the need for hospitalization and surgery. Different reasons can explain this discrepancy: firstly, the selection criterion for the randomized trials excluded patients with an imminent need for surgery and therefore contains a selection bias. Secondly, not all patients respond to anti-TNF therapy and the presence of a stricture and/or penetrating disease at the time of diagnosis is highly predictive for the need of surgery and

conservative treatment failure. Thirdly, most experience has been gained with the use of infliximab. The immunogenicity of the drug will lead to a substantial loss of response over time[41,42]. Finally, a genuine resistance (irrespective to immunogenicity) to anti-TNF drugs has been observed. This will lead to a drop-out of 10% of patients per year. Results of recent top-down strategies clearly demonstrate the beneficial effect of early "aggressive" treatment of luminal inflammation[43]. The medium-term benefits with regard to clinical remission and the need for surgery seem to indicate that a disease modification can indeed be obtained in a subset of patients. This concept therefore needs to be further explored and implemented into clinical practice.

The face of surgery has also evolved over time. Today, most patients can benefit from a minimally invasive approach (laparoscopy and single site laparoscopy). Furthermore, isoperistaltic stricturoplasty has demonstrated its safety and long-term efficacy in the treatment of long strictures of the small bowel and reduces the ultimate risk for intestinal failure[44]. The implementation of enhanced recovery protocols further expedites patient rehabilitation after surgery. These aspects open a more attractive alternative to protracted medical treatment. Surgical-recurrence free survival at 5 years after primary ileocaecal resection is as high as 91% (own unpublished data). However early endoscopic and symptomatic disease recurrence hampers the enthusiasm for an early surgical approach. In patients with anorectal CD, surgery remains an essential, and often first, step in the treatment algorithm. Anal examination under anesthesia and drainage of perianal abscess precedes maintenance medical treatment. This combined approach is essential to safeguard anorectal function in the maximum number of patients, and to avoid definitive proctocolectomy and stoma formation.

The introduction of anti-TNF agents in the 1990s changed treatment algorithms in CD and has the potential to alter the natural history of the disease. Randomized data show a significant decrease in the development of complications and the need for surgery. Sustained mucosal healing seems a good predictor for fewer complications and surgery in the long-term. No reduction in the need for surgery has been documented in population-based surveys. This discrepancy is multifactorial. Further evolution and implementation of top-down treatment strategies should eventual lead to a genuine reduction in the need for surgery. For the time being, surgery still plays a pivotal role in a large subset of patients in order to obtain long-term disease remission and improvement of patient quality of life. However, the evolving concept of disease modification will certainly alter the role and need for surgery in the future. Optimal treatment of CD remains a joint effort of dedicated physicians and surgeons.

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TOPIC HIGHLIGHT

Giovanni Latella, MD, *Series Editor*

Role of surgery in severe ulcerative colitis in the era of medical rescue therapy

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Abstract

Despite the growing use of medical salvage therapy, colectomy has remained a cornerstone in managing acute severe ulcerative colitis (ASC) both in children and in adults. Colectomy should be regarded as a life saving procedure in ASC, and must be seriously considered in any steroid-refractory patient. However, colectomy is not a cure for the disease but rather the substitution of a large problem with smaller problems, including fecal incontinence, pouchitis, irritable pouch syndrome, cuffitis, anastomotic ulcer and stenosis, missed or de-novo Crohn's disease and, in young females, reduced fecundity. This notion has led to the widespread practice of offering medical salvage therapy before colectomy in most patients without surgical abdomen or toxic megacolon. Medical salvage therapies which have proved effective in the clinical trial setting include cyclosporine, tacrolimus and infliximab, which seem equally effective in the short term. Validated predictive rules can identify a subset of patients who will eventually fail corticosteroid therapy after only 3-5 d of steroid therapy with an accuracy of 85%-95%. This accuracy is sufficiently high for initiating medical therapy, but usually not colectomy, early in the admission without delaying colectomy if required. This approach has reduced the colectomy rate in ASC from 30%-70% in the past to 10%-20% nowadays, and the mortality rate from over 70% in the 1930s to about 1%. In general, restorative proctocolectomy (ileoanal pouch or ileal pouch-anal anastomosis), especially the J-pouch, is preferred over straight pull-through (ileo-anal) or ileo-rectal anastomosis, which may still be considered in young females concerned about infertility. Colectomy in the acute severe colitis setting, is usually performed in three steps due to the severity of the inflammation, concurrent steroid treatment and the generally reduced clinical condition. The first surgical step involves colectomy and constructing an ileal stoma, the second - constructing the pouch and the third - closing the stoma. This review focuses on the role of surgical treatment in ulcerative colitis in the era of medical rescue therapy.

Key words: Acute severe ulcerative colitis; Colectomy; Corticosteroids; Cyclosporine; Infliximab; Tacrolimus

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INTRODUCTION

Acute severe colitis (ASC) is one of the few emergencies in gastroenterology. The mortality rate from ASC dropped from over 70% in 1933 to 20%-25% in the 1950s when the importance of timely urgent colectomy was first recognized[1,2]. Subsequently, the mortality rate was further reduced to 7% with the introduction of corticosteroids, and eventually to about 1% nowadays[3-5].

Truelove *et al*[3], in their landmark clinical trial in 1955, defined severe disease as the passage of at least six daily bloody stools, erythrocyte sedimentation rate > 30, temperature > 37.8 ℃, pulse rate > 90/min and hemoglobin < 10.5 g/dL. These criteria remain the most common classification of ASC in adults. In a systematic review of cohort studies on ASC, 20 of 29 studies used the Truelove and Witts classification[5]. However, of these 20 studies, 12 required the fulfillment of all of the five items, and 8 applied various more liberal modifications. More recently, the European Crohn's and Colitis Organization (ECCO) issued guidelines on managing ASC defining severe attack as ≥ 6 bloody diarrhea per day with at least *one* of the other four bullets[6]. In children, severe disease has been robustly defined using the Pediatric Ulcerative Colitis Activity Index (PUCAI) with a score of at least 65 points yielding high sensitivity and specificity[7,8]. Pediatric onset ulcerative colitis (UC) is often more extensive than in adults[9], and since disease severity has been consistently associated with disease extent, children are especially susceptible to refractory severe attacks. Nearly one third of children with ulcerative colitis will experience at least one severe exacerbation before turning into adult care[10].

PREDICTING THE NEED FOR SALVAGE THERAPY

The only factor associated with a major surgical complication among 80 adults who underwent urgent colectomy in Oxford for ASC, was longer duration of medical therapy before colectomy[11]. It is of great importance to recognize those who are likely to fail intravenous corticosteroid early during the admission and to introduce timely rescue therapy. This approach may reduce morbidity and mortality and avoid futile toxic medical therapy.

Several clinical predictive indices have been proven to perform well in identifying those who require salvage therapy after only 3-5 d of admission. In a prospective study, Travis *et al*[12] from Oxford suggested that a stool frequency of > 8/d or 3-8/d and C-reactive protein (CRP) > 45 mg/dL on the third day of corticosteroid therapy should be sufficient for initiating rescue therapy. Lindgren *et al*[13] developed the fulminant colitis index (n = 97) based on the same variables as the Oxford index - stool frequency/d + 0.14 × CRP mg/L. Ho *et al*[14] based the Scottish index on stool frequency, presence of colonic dilatation, and hypoalbuminemia. Others have also shown some predictive ability of their indices[15,16]. In children, a predictive rule based on the PUCAI score at days three and five of steroid therapy has been validated[10,17] and incorporated in the combined ECCO and European Society for Paediatric Gastroenterology Hepatology and Nutrition recent guidelines on pediatric ASC[8]. A PUCAI score of > 45 points on day 3 should dictate planning of second-line therapy and > 65-70 points on day 5 should prompt execution of the planned therapy. All aforementioned indices are based on the consistently reproduced fact that the likelihood for responding to medical corticosteroids is inversely associated with the degree of disease severity even before treatment has been initiated.

Despite the significant improvement in patient care with the implementation of indices-based management schemes, their accuracy is still imperfect. The positive predictive value (PPV) of the Oxford index in predicting steroid failure is 85% for colectomy and the sensitivity and specificity of the Scottish index is 85% and 75%, respectively. The PPV of the fulminant colitis index at a cutoff score of > 8 at day 3 is 69%-72%[13,18]. The PUCAI successfully identifies those for whom the likelihood of failing corticosteroids is 92%. These indices should be perceived as accurate enough for initiating salvage medical therapy (i.e., infliximab or calcineurin inhibitors) but probably not for early irreversible colectomy (Table 1).

SALVAGE MEDICAL THERAPY

Medical rescue therapy should be utilized as the first-line treatment in ASC before colectomy in most corticosteroid-failed patients who do not present with surgical abdomen or toxic megacolon. During the last 20 years, cyclosporine has been widely used[19] and more recently also infliximab[18], and tacrolimus[20] . These medications reduced the short-term colectomy rate from 30%-70%[3,5,21,22] to approximately 10%-20% nowadays[17,23]. The increasing use of second-line medical therapy before colectomy has been based on the high effectiveness of these drugs and the notion that colectomy is not a cure for the disease, but rather the substitution of one large problem with several smaller problems.

Experience with cyclosporine showed that, although the short-term response rate reaches 70%-80%[5,9], approximately 50% of responders will eventually require colectomy when the drug is discontinued, typically after 4 mo[5,21,24-26]. The likelihood of colectomy is reduced if cyclosporine is used as a bridging medication to thiopurines[27]. The other calcineurin inhibitor, tacrolimus (FK-506), has recently been proved effective as a second-line regimen in the clinical trial setting, while aiming at high trough levels of 10-15 ng/mL[20]. It seems that tacrolimus is as effective as cyclosporine for salvage therapy in ASC, both in adults and children[21,28,29]. Tacrolimus, a more expensive medication, has a better bioavailability than cyclosporine, allowing for oral treatment. The toxicity profile is more appealing but there are fewer published studies to support its use.

Table 1 Prediction rules for corticosteroid failure in patients with acute severe ulcerative colitis

Prediction rule	Study	Measure	Prediction accuracy
Oxford index	Travis *et al*[12], prospective; Turner *et al*[10], retrospective	Stool frequency of > 8/d or 3-8/d and CRP > 45 mg/L (on day 3 of IVCS)	Adults: PPV = 85% Children: Sens = 38%, Spec = 100%, PPV = 88%, NPV = 75%
Swedish index (the fulminant colitis index)	Lindgren *et al*[13], retrospective; Järnerot *et al*[18], prospective; Turner *et al*[10], retrospective	CRP mg/L × 0.14 + daily stool frequency (cutoff > 8 on day 3 of IVCS)	Adults: Sens = 78%, Spec = 81%, PPV = 69%-72% Children: Sens = 64%, Spec = 92%, PPV = 88%, NPV = 75%
Seo index	Seo *et al*[15], retrospective; Turner *et al*[10], retrospective	60 × bloody stool + 13 × bowel movements + 0.5 × ESR - 4 × Hb - 15 × albumin + 200	Adults (cutoff > 180 on day 7 of IVCS): PPV = 52%, NPV = 97% Children: (cutoff > 240 on day 5 of IVCS): Sens = 27%, Spec = 93%, PPV = 80%, NPV = 56%
Scottish index	Ho *et al*[14], prospective	The score (0-9) includes: stool frequency, presence of colonic dilatation, and albumin level (cutoff > 4 on day 3 of IVCS)	Adults: Sens = 85%, Spec = 75%
PUCAI	Turner *et al*[10], retrospective; Turner *et al*[17], prospective	The score (0-85) includes: stool frequency and consistency, presence of blood, nocturnal stools, activity level, and abdominal pain	Children: PUCAI > 45 on day 3 of IVCS: Sens = 92%-93%, NPV = 88%-94% PUCAI > 70 on day 5: Sens = 35%-44%, Spec = 93%-100%, PPV = 87%-100%, NPV = 63%-79%

NPV: Negative predictive value; PPV: Positive predictive value; Hb: Hemoglobin; ESR: Erythrocyte sedimentation rate; IVCS: Intravenous corticosteroids; Spec: Specificity; Sens: Sensitivity; CRP: C-reactive protein; PUCAI: Pediatric Ulcerative Colitis Activity Index.

Infliximab has been established as an effective regimen for moderate to severe ulcerative colitis, including ASC. The ACT-1 and ACT-2 randomized controlled trials assessed the ability of infliximab to induce and maintain remission in moderate to severe ulcerative colitis[30,31]. A total of 728 adult patients received placebo or infliximab (5 or 10 mg/kg) through week 46 (ACT-1) or 22 (ACT-2). In the ACT-1 and 2 trials respectively, 61% and 69% of infliximab-treated subjects had a short-term clinical response compared with 29% and 37% of those who received placebo. In steroid-refractory ASC, infliximab is effective as salvage medical therapy in approximately 70%-80% of children and adults, reducing short- and long-term colectomy rate[17,18,32]. In the Jarnerot trial, the colectomy-free rate was 12/24 (50%) after 3 years[33]. Combining the data of both ACT trials has shown that the 1-year colectomy rate in the infliximab-treated arm was 10% *vs* 17% in the placebo arm[34]. It should be emphasized that the ACT trials did not include patients who were refractory to intravenous steroid in the setting of ASC.

In the recent steroid-refractory severe attacks of ulcerative colitis trial, 116 adults with ASC who did not respond to a 5-d course of intravenous steroids were randomized to receive intravenous cyclosporine or infliximab using standard doses and protocols, both combined with azathioprine[35]. Both the 7-d response rate (85.4% *vs* 85.7%) and treatment failure rate through day 98 (60% *vs* 54%) were similar between the cyclosporine and the infliximab arms, suggesting that the two regimens are equally viable alternatives to colectomy in steroid-refractory ASC. Similar effectiveness has also been suggested in a systematic review of non-randomized studies in children[21]. In contrast, a nonrandomized study showed that 52% of patients receiving cyclosporine proceeded to colectomy by discharge, *vs* 18% of those administered infliximab[36].

SURGERY

Although, in general, medical rescue therapy should be regarded as the first-line treatment in steroid-refractory ASC, colectomy is still a cornerstone of the management scheme. Colectomy is indicated in ASC not responding to medical therapy, toxic megacolon, perforation, and uncontrolled colorectal bleeding (rare)[37,38]. Colectomy in steroid-refractory ASC cases may be considered before medical salvage therapy in chronic active UC previously resistant to thiopurines and infliximab, since no maintenance therapy would be available after discontinuing the calcineurin inhibitor. Surgery is usually the preferred therapeutic option in patients with toxic megacolon, a life threatening event. However, a 24-48 h trial of conservative treatment (i.e., bowel rest, broad spectrum antibiotics and rectal tube) may be cautiously attempted in the non-severe cases in specialized centers only while under intense monitoring. Sequential therapy of calcineurin inhibitors followed by infliximab or vice versa may be successful in approximately 25%-40% of adult patients, but is associated with significant morbidity and even mortality[39-42]. Therefore, most recommend timely referral for colectomy after failing one medical salvage therapy, rather than attempting another regimen[6,8]. Expected response to calcineurin inhibitors and infliximab is roughly 1-2 wk and colectomy should not be withheld in non-responders.

Although in the past ileoanal straight anastomosis has been the procedure of choice, now the ileal pouch-anal anastomosis (IPAA) (also known as "restorative proctocolectomy") is the most commonly practiced surgery, also in children. In some centers, ileo-rectal anastomosis is practiced but limited data are available to support this surgery. High early failure rates have been reported with this surgery and a life-long follow-up of the retained rectal stump is required. The ileoanal

pouch procedures are likely superior to the straight pull through (i.e., ileoanal anastomosis) as they are associated with a lower early stool frequency and better long-term continence while maintaining acceptable early complication rates. However, the IPAA procedure is associated with pouchitis in approximately 45%-60%[43-45], of whom 60% will suffer from recurrent episodes and 5%-10% will develop chronic pouchitis[45]. The probability of pouch failure has been found to be 9% at 10 years[43]. Daytime and nighttime incontinence occurred in 7%-10% and 12%-24% of patients, respectively, over a 10-15 year period[43,44]. The risk for female infertility after IPAA seems to be a major concern with an increase from approximately 10% in the average population to 25%-30%[8,46]. The role of ileo-rectal anastomosis is controversial, but may be considered in females who are primarily concerned about the reduced fecundity associated with IPAA. The apparent advantages of the IPAA procedure must be seriously balanced against the potential adverse events which should be discussed openly with the families.

IPAA can be performed in one, two or three stages. A two-stage procedure (colectomy with pouch construction and a temporary protecting loop ileostomy to be closed in the second stage) is the most frequent procedure in stable ambulatory patients. The one step procedure (restorative proctocolectomy without protecting ileostomy) may be safe in selected ambulatory patients without any risk factors (e.g., steroids treatment, malabsorption and hypoalbuminemia) in highly trained centers[8]. A three-stage approach (colectomy with temporary ileostomy in the first stage, pouch construction in the second stage and ultimately ileostomy closure) should be performed in patients with steroid-refractory acute severe colitis, those on high dose steroids and/or suffering from malnutrition, and those in whom Crohn' s disease has not been excluded[8] . With any chosen procedure, a laparoscopic-assisted procedure is feasible and safe[47,48], also in children[49,50]. The overall complication rate was higher in open surgery, compared with laparoscopic surgery (55% *vs* 39%, $P = 0.004$)[48] with longer hospital stay. Patients who had an ileal pouch created through the laparoscopic approach had fewer occurrences of pouchitis[49]. There were no significant differences between the two groups regarding daytime and night continence, or sexual function[47].

In a meta-analysis, pouch failure rate was found to be 4.3% (95% CI: 3.5-6.3) and pelvic sepsis 7.5% (95% CI: 6.1-9.1)[51]. Pouch failure was lower by 2.5% in recent studies *vs* those published prior to 2000. Functional outcome remained stable over time, with a 24-h defecation frequency of 5.9 (95% CI: 5.0-6.9), regardless of the technical aspects of the surgery[51]. Preoperative steroid therapy (> 20 mg in adults), hypoalbuminemia and malnutrition are associated with increased surgical complications[52]. Pre-operative high dose steroids and probably also infliximab[53] are associated with increased surgical complications (especially in combination with other immune suppressants), while thiopurines and calcineurin inhibitors are not.

AMBULATORY SURGERY

The most frequent indication for colectomy in ambulatory children with UC is chronic ongoing disease, at times- steroid dependent, whereas in adults- dysplasia is also a common indication[54]. The points outlined above for ASC should also be followed in the decision-making of elective colectomy. In general, thiopurines and infliximab should be strongly considered in most cases before referral to colectomy in ambulatory mild-moderate UC. While cyclosporine should be initially administered in the hospital setting only, tacrolimus may be used in selected ambulatory patients as a bridge to thiopurines. In those losing response to infliximab, adalimumab may be considered before colectomy, given the recent evidence in adults showing its moderate effectiveness in ambulatory UC[55,56]. Colectomy should be discussed as a viable alternative in children who suffer from ongoing symptoms despite multiple immunosuppressive medications, especially in steroid dependency.

CONCLUSION

Colectomy, a potentially lifesaving procedure in ASC, is associated with several long-term unwanted consequences. On the other hand, medical rescue therapy, including cyclosporine, tacrolimus and infliximab, are highly effective in steroid-refractory ASC. Therefore, medical salvage therapy should be offered before colectomy in most patients who do not present with surgical abdomen or toxic megacolon. Validated predictive rules can identify a subset of patients who will eventually fail corticosteroid therapy after only 3-5 d of steroid therapy with an accuracy of 85%-95%. This accuracy is sufficiently high for initiating medical therapy early in the admission without delaying colectomy if required. Families may elect to proceed to early colectomy before attempting medical rescue therapy, especially in chronic active disease. Therefore, whenever considering second-line medical therapy, colectomy should always be openly discussed. In patients failing one medical rescue therapy, colectomy should be regarded as the next therapeutic step.

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TOPIC HIGHLIGHT

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Colorectal cancer in inflammatory bowel disease: What is the real magnitude of the risk?

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Abstract

The association between inflammatory bowel disease (IBD) and colorectal cancer (CRC) has been recognised since 1925 and still accounts for 10%-15% of deaths in IBD. IBD-associated CRC (IBD-CRC) affects patients at a younger age than sporadic CRC. The prognosis for sporadic CRC and IBD-CRC is similar, with a 5-year survival of approximately 50%. Identifying at risk patients and implementing appropriate surveillance for these patients is central to managing the CRC risk in IBD. The increased risk of colorectal cancer in association with IBD is thought to be due to genetic and acquired factors. The link between inflammation and cancer is well recognised but the molecular biology, immune pathobiology and genetics of IBD-CRC are areas of much ongoing research. This review examines the literature relating to IBD-CRC, focusing on the incidence of IBD-CRC and examining potential risk factors including age at diagnosis, gender, duration and extent of colitis, severity of inflammation, family history of sporadic CRC and co-existent primary sclerosing cholangitis (PSC). Confirmed risk factors for IBD-CRC are duration, severity and extent of colitis, the presence of co-existent PSC and a family history of CRC. There is insufficient evidence currently to support an increased frequency of surveillance for patients diagnosed with IBD at a younger age. Evidence-based guidelines advise surveillance colonoscopy for patients with colitis 8 to 10 years after diagnosis, with the interval for further surveillance guided by risk factors (extent of disease, family history of CRC, post-inflammatory polyps, concomitant PSC, personal history of colonic dysplasia, colonic strictures). There is a move away from using random colonic biopsies towards targeted biopsies aimed at abnormal areas identified by newer colonoscopic techniques (narrow band imaging, chromoendoscopy, confocal microendoscopy).

Key words: Colorectal cancer; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Risk

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INTRODUCTION

Crohn[1] first described colorectal cancer (CRC) in association with inflammatory bowel disease (IBD) in 1925 and colorectal cancer still accounts for 10%-15% of deaths in patients with IBD[2]. The incidence of IBD

has two peaks; between 15 and 30 years, and between 50 and 80 years[3]. Due to this younger age peak, the mean age for developing IBD-associated CRC (IBD-CRC) is lower than that seen in sporadic CRC[4]. A meta-analysis of 116 studies found the mean age of IBD-CRC diagnosis to be 43.2 years[5]. Lakatos *et al*[6] found the average age of IBD-CRC diagnosis to be 10 to 15 years younger than sporadic CRC in Eastern Europe (50.9 years *vs* 62.2 years). The prognosis for sporadic CRC and IBD-CRC is similar with a 5-year survival of approximately 50%[7]. Identifying at risk patients and implementing appropriate surveillance for these patients is central to managing the CRC risk in IBD.

The increased risk of colorectal cancer in association with IBD is thought to be due to genetic and acquired factors[8]. The link between inflammation and cancer is well recognised but the molecular biology, immune pathobiology and genetics of IBD-CRC are areas of much ongoing research. The role of the immune system for both tumour promotion and prevention is being examined. Studies indicate that immune cells are important in tumour promotion, with leucocyte infiltration and inflammatory mediators found in association with tumours[9,10].

Genome-wide association studies have now identified approximately 99 susceptibility loci/genes relating to IBD. The better understanding of the genetics of IBD gives us more insight into disease pathogenesis. Some of the key themes identified are the role of interleukin (IL)-23/IL-17 signalling in IBD, defective barrier function in ulcerative colitis (UC) and defective processing of intracellular bacteria in Crohn's disease[11]. Overlap of these loci/genes is seen with many diseases including CRC: E-cadherin (CDH1) has been associated with CRC[12], UC[13], and possibly Crohn's disease[14].

MOLECULAR BIOLOGY OF IBD-CRC

IBD-CRC and sporadic CRC both have a dysplasia-cancer sequence and require multiple mutations to result in carcinoma[7]. Sporadic CRC usually occurs as the end point of the adenoma-carcinoma sequence. Multiple molecular alterations occur within this sequence: chromosomal instability, microsatellite instability, hypermethylation[15,16]. However, there are often differences in the timing and frequency of these events between sporadic and IBD-CRC. The molecular and genetic alterations occur more rapidly in IBD-CRC and in an unconventional sequence[17]. It is suggested that the inflammation occurring in colitis results in a cascade of abnormal epithelial proliferation in addition to the genetic alterations that occur[18]. In sporadic CRC, loss of the adenomatous polyposis coli gene function occurs early and p53 mutations occur late. The timing of these events is frequently reversed in IBD-CRC. It is also notable that in IBD-CRC, p53 mutations may be found within non-dysplastic mucosa. This is markedly different from sporadic CRC where p53 mutations are seen in morphologically aggressive lesions[19].

The dysplasia-cancer sequence is a useful concept but it is more complex in IBD-CRC. There is not always a clear, stepwise transition from normal, through low and high grade dysplasia (HGD), to cancer[17]. However, low grade dysplasia (LGD) can clearly progress to more advanced lesions and there is varying evidence as to the size of this risk. The 5-year cumulative risk has been shown to be as high as 33%-54% in a number of studies[20-23]. This is in contrast to a 2%-10% risk of CRC over 10 years found by other groups[24,25].

Cancer can occur without preceding detection of dysplasia, and LGD can regress or progress directly to cancer without the intermediate HGD[26]. The high rate of synchronous and metachronous cancers associated with finding high grade dysplasia in biopsies usually leads to proctocolectomy[27]. In a review of 10 prospective trials, 42% of patients undergoing colectomy for HGD had a synchronous CRC[28]. Low grade dysplasia is an independent risk factor for CRC and often found in flat lesions which are difficult to see endoscopically. This is in contrast to sporadic CRC where dysplasia occurs within raised polypoid lesions. The concern is that flat LGD may be accompanied by HGD or cancer elsewhere in the colon. Studies suggest that 16%-27% of patients undergoing colectomy for LGD have synchronous CRC[20,22,28]. Opinions differ as to how LGD should be managed: colectomy versus colonoscopic surveillance. Informed patient choice is important here. If surveillance is pursued colonoscopy should be repeated in 3 to 6 mo. The presence of multifocal LGD is a much stronger argument for colectomy.

QUANTIFYING THE RISK

It is important to quantify the risk of CRC in association with IBD. The reported risk varies widely between studies. This is partly due to the different methodology used in studies. Data comes from a mixture of tertiary referral centres, district general hospitals and population-based studies. Information from tertiary centres is likely to include patients with severe disease who are at greater risk of IBD-CRC. Early studies included patients who had already been referred with a diagnosis of CRC and those admitted to hospital with IBD, rather than gold-standard population-based studies which have a lower proportion of patients with severe or extensive colitis.

Eaden *et al*[5] performed a meta-analysis of 116 studies including 54 478 UC patients with 1698 cases of IBD-CRC. The analysis included studies from a wide variety of centres: tertiary referral centres, population-based studies and hospital-based centres, and from different geographical areas. Studies from the United Kingdom and United States found a higher incidence [4 and 5 per 1000 person-years duration (pyd), respectively] than those from Scandinavia (2 per 1000 pyd). They found the overall prevalence of CRC in UC to be 3.7%, increasing to 5.4% in those with pancolitis.

Ekbom *et al*[29] undertook a population-based cohort

study of 3117 patients with UC who were diagnosed between 1922 and 1983. Ninety one patients were found to have IBD-CRC giving a standardised incidence ratio of 5.7 (95% CI: 4.6-7.0) as compared with the expected incidence of CRC in the general population.

Söderlund *et al*[30] undertook a population-based study of 7607 patients with IBD who were diagnosed between 1954 and 1989 for a total of 198 227 person-years. A total of 196 cases of CRC occurred in 188 patients, giving an overall incidence of 85 (95% CI: 82-109) cases per 100 000 person-years. This corresponds to a standardised incidence ratio of 2.3 (95% CI: 2.0-2.6) as compared with the general population.

Other population-based studies have suggested a much lower risk of IBD-CRC. Palli *et al*[31] followed 689 patients with UC over 14 years (1978-1992) and found 10 cases of IBD-CRC, equating to an annual crude incidence of 0.13%. A study in Olmsted County followed 378 patients with UC for a total of 5567 person-years (1940-2004) and found 6 cases of IBD-CRC. They calculated the annual crude incidence to be 0.10% and the cumulative risk of CRC at 30 years to be as low as 2%. They found no statistically significant increase in the standardised mortality ratio (SMR) for CRC between IBD and non-IBD populations and concluded that the risk of CRC is only increased in patients with extensive colitis[32]. Bernstein *et al*[33] retrospectively examined 2672 patients with UC over a total of 19 665 person-years between 1984 and 1997. They found the annual risks to be 0.16% for colon cancer and 0.06% for rectal cancer.

Rutter *et al*[20] followed 600 patients with extensive UC (as shown by barium enema or colonoscopy) for 5932 person-years as part of a colonoscopic surveillance programme (1970-2001). Data was gathered prospectively. Ninety-one patients (163 episodes) were found to have dysplasia or CRC. They calculated the cumulative probability of IBD-CRC to be 7.6% and a decreasing incidence over the period studied.

A Hungarian population-based study followed 723 UC patients for a total of 8564 person-years (1974-2004) and found 13 cases of IBD-CRC. They calculated the cumulative risks according to disease duration: 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years[6]. Hungary has a high rate of sporadic CRC and a low rate of colectomy for non-CRC reasons; factors which might have been expected to result in a higher rate of CRC.

Winther *et al*[34] followed 1160 patients with UC over 22 290 person-years (1962-1987) and found 13 cases of IBD-CRC, giving an annual crude incidence of 0.06% and cumulative risk of 2.1% at 30 years. They found no statistically significant increase in the SMR for CRC between IBD and non-IBD populations. This study is from Denmark where the colectomy rate is one of the highest in the world: a fact that may affect the results and underestimate the risk of IBD-CRC.

The increased risk of CRC in UC has been long established. More recently, data has shown that Crohn's colitis carries a similar magnitude of risk for the same disease extent. A Canadian cohort study matched a population-based IBD database to a cancer registry in North America between 1984 and 1997. There were 2857 cases of Crohn's disease and 2672 of UC. There was an increased incidence of CRC for patients with Crohn's [risk ratio (RR) 2.64; 95% CI: 1.69-4.12] or UC (RR 2.75; 95% CI: 1.91-3.97) as compared to the general population but no statistically significant difference between the two IBD diagnoses[33]. They found the risk of rectal cancer to be increased in UC (RR 1.90; 95% CI: 1.05-3.43) but not in Crohn's colitis (RR 1.08; 95% CI: 0.43-2.70). A limitation of this study was the lack of definition of disease site or extent.

Ekbom *et al*[35] also studied the risk of CRC in Crohn's disease. In a cohort study of 1655 patients in Sweden, patients with terminal ileal Crohn's had the same risk of CRC as the general population but those with colonic Crohn's had a RR of 5.6 (95% CI: 2.1-12.2).

CHANGING INCIDENCE OVER TIME

Eaden *et al*[5] examined how the incidence of IBD-CRC is changing over time by plotting the cancer risk against the mid-point for each study. Although the incidence had increased between 1995 and 2001 the change was not statistically significant (slope 0.003, $P = 0.80$).

Rutter *et al*[20] looked at the changing incidence of CRC over time within their 30-year colonoscopic surveillance program. They found a statistically significant decrease in CRC incidence ($r = -0.40$, $P = 0.04$), specifically for cancers proximal to the splenic flexure ($r = -0.54$, $P = 0.005$) and early cancers since 1975 (Dukes' A or B; $r = -0.4$, $P = 0.04$). However, there was not a statistically significant change in incidence for distal cancers ($r = 0.11$, $P = 0.59$) or advanced cancers (Dukes' C or D; $r = -0.05$, $P = 0.79$).

Jess *et al*[32] examined a population-based cohort from Minnesota diagnosed between 1940 and 2001. They followed 692 patients with IBD for a total of 10 470 person-years. They analysed the incidence of CRC and the year that IBD was diagnosed. All their patients were diagnosed with CRC before 1980. The standardised incidence ratio for CRC diagnosed before 1980 was 1.6 (95% CI: 0.6-3.4) in comparison to 0 for those diagnosed after 1980. However, this difference between calendar periods was not statistically significant. They suggested that use of maintenance therapy and surveillance colonoscopy may be responsible for the absence of CRC seen in this cohort.

Söderlund *et al*[30] examined the relative risk of CRC across calendar periods in their population-based study of 7607 patients with IBD who were diagnosed between 1954 and 1989. This cohort was followed up until 2004. After adjusting for type and extent of IBD, gender, age, and time since IBD diagnosis, the RRs were 1.7 (95% CI: 0.6-4.4) from 1960 to 1969, 1.3 (95% CI: 0.7-2.6) from 1970 to 1979, 1.2 (95% CI: 0.7-2.2) from 1980 to

1989, 1.1 (95% CI: 0.7-1.8) from 1990 to 1999, and 1.0 from 2000-2004. Although there was a trend towards decreasing incidence of CRC this did not reach statistical significance.

RISK FACTORS

Duration of colitis

A longer duration of colitis is associated with an increased risk of IBD-CRC; it is relatively rare before 8 years of colitis. However, Lutgens *et al*[36] conducted a retrospective, nationwide database search to identify patients with IBD-CRC in the Netherlands between 1990 and 2006. For the 149 patients identified, they calculated the time interval between the diagnoses of IBD and cancer. They found that 22%-28% of patients developed cancer before the starting points for surveillance (8-10 years for extensive colitis and 15-20 years for left-sided disease). When the time interval from onset of symptoms to CRC diagnosis was used, 17%-22% would have developed cancer prior to surveillance endoscopy.

Identifying when the risk increases is central to guiding timing of surveillance strategies. Patients may also have colitis for a time before the diagnosis is made. This means they are exposed to cancer risk for a longer period if diagnosis is delayed.

In the meta-analysis by Eaden *et al*[5], 41 studies reported colitis duration and 19 of these stratified duration into 10 year intervals. The overall incidence of IBD-CRC in any patient with UC was 3 per 1000 pyd. The cumulative incidence of IBD-CRC in patients with UC was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease. In the 61 studies that reported duration of colitis at the time of IBD-CRC diagnosis the mean was 16.3 years (95% CI: 15.0-17.6).

Lakatos *et al*[6] retrospectively examined data relating to 723 patients within their 30-year database of IBD patients. They found 13 cases of IBD-CRC in 8564 person-years. They examined the incidence of CRC according to disease duration and found the cumulative risk to be 0.6% at 10 years (95% CI: 0.2-1.0), 5.4% at 20 years (95% CI: 3.7-7.1), and 7.5% at 30 years (95% CI: 4.8-10.2).

The prospective surveillance data from Rutter *et al*[20] found the median duration of UC at diagnosis of CRC to be 23.5 years (range 11-48 years). The cumulative incidence of CRC was 0% at 10 years, 2.5% at 20 years, 7.6% at 30 years, 10.8% at 40 years, and 13.5% at 45 years (from onset of colitis). This lower incidence of IBD-CRC is particularly interesting given that the data comes from a specialist centre with a low colectomy rate and might have been expected to show a higher incidence. Data being obtained from a surveillance programme may in itself be responsible for the lower incidence of CRC, and regular colonic examination should prompt treatment measures to gain disease control.

Age at diagnosis

There is conflicting evidence as to whether younger age at diagnosis of IBD is an independent risk factor for IBD-CRC. This evidence is not easy to evaluate as children tend to have more extensive and severe colitis than those diagnosed as adults, and younger people have the potential for longer colitis duration, which is itself a risk factor.

An important question is whether patients studied actually develop colitis at a younger age or whether they are diagnosed late after a period of undiagnosed inflammation. Eaden *et al*[5] included 21 studies in their meta-analysis that examined age at onset of UC (over 20 years old). They excluded studies that reported the age at diagnosis of UC due to potential delay in diagnosis. They found a negative trend between younger age at onset (in adulthood) and increased risk of CRC but this was not statistically significant (z = -1.61, P = 0.11). They also analysed 12 studies looking at the incidence of IBD-CRC in children with UC (average age at onset of UC was 10 years old), only 5 of which commented on the duration of follow up. They found the overall incidence to be 6 per 1000 pyd and the cumulative risk of CRC to be 5.5% at 10 years, 10.8% at 20 years, and 15.7% at 30 years; higher than the corresponding rates for adults. However, these studies did not report incidence according to 10 yearly intervals so they assumed the log incidence rate to be constant. The small number of studies in children necessitates cautious interpretation of results.

Ekbom *et al*[29] conducted a population-based cohort study of 3117 patients who were diagnosed with UC between 1922 and 1983 in Sweden. CRC was identified in 91 patients. They found age at diagnosis to be an independent risk factor for CRC. After adjusting for the extent of disease, they found the relative risk of CRC decreased by about half (adjusted standardized incidence ratio = 0.51; 95% CI: 0.46-0.56) for each increase in age group at diagnosis (under 15 years, 15-29 years, 30-39 years, 40-49 years, 50-59 years, and over 60 years). For those with extensive disease, after 35 years of disease, the cumulative risk for IBD-CRC was 40% if diagnosed under 15 years and 25% if diagnosed between 15 and 39 years of age.

Other studies have not confirmed this association. Greenstein *et al*[37] found that the CRC risk was higher in patients diagnosed with IBD above 30-40 years of age compared with those diagnosed below 20 years old. Data from the 30-year study of Rutter *et al*[20] showed that patients who developed CRC had a higher median age of onset of disease than those not developing cancer. This suggests that early onset is not an independent risk factor for IBD-CRC. Winther *et al*[34] found the time between onset of colitis and the development of IBD-CRC to be the same in young and old patients.

Karvellas *et al*[38] undertook a retrospective audit of adult patients with UC who were diagnosed with CRC between 1991 and 2002 in Edmonton, Alberta. They found that patients diagnosed with UC over the age of 40 years developed CRC more quickly than younger patients. The median disease duration at the time of CRC

diagnosis was 22 years in patients under 40 years, and 10 years for those over 40 years [odds ratio (OR): 11.5; 95% CI: 2.41-20.16; $P = 0.00029$].

Extent of colitis

The greater the disease extent, the greater the risk of CRC. Patients with proctitis and proctosigmoiditis are at the lowest risk, left-sided colitis carries moderate risk and those with pan-colitis are at the highest risk of CRC[39,40].

How we measure the extent of disease is important; macroscopic versus radiological versus histological. Ekbom *et al*[29] assessed IBD-CRC risk in UC according to extent of disease; proctitis versus left-sided colitis versus pancolitis. The extent was determined from barium enema and colonoscopy reports. The relative risk for CRC was 1.7 for proctitis, 2.8 for left-sided colitis, and 14.8 for pancolitis, as compared with the general population.

Söderlund *et al*[30] examined the risk of CRC according to extent of colitis in their population-based study. They found the relative risks of CRC to be 2.7 for all patients with UC (95% CI: 2.3-3.2), 5.6 for pancolitis (95% CI: 4.0-4.7), 2.1 for Crohn's colitis (95% CI: 1.2-3.4) and 1.7 for proctitis (95% CI: 1.2-2.4).

Severity of inflammation

IBD-CRC is thought to occur in the context of inflammation and anti-inflammatory treatments are felt to decrease the risk of CRC. Recent studies have focused on this association.

Rutter *et al*[20] conducted a case-control study of patients with colorectal neoplasia identified within a surveillance program between 1988 and 2002 ($n = 68$). They found a significant correlation between both colonoscopic (OR: 2.5; $P < 0.001$) and histological (OR: 5.1; $P < 0.001$) inflammation and the risk of neoplasia. When multivariate analysis was performed, histological inflammation remained a significant risk factor (OR: 4.7; $P < 0.001$). In a follow-up study from this work, Rutter *et al*[41] found that mucosal healing may decrease the risk of neoplasia; macroscopically normal mucosa appears to return the CRC risk to that of the general population.

Gupta *et al*[42] retrospectively studied a cohort of 418 patients undergoing colonoscopic surveillance for UC. They found a significant relationship between histological inflammation over time and progression to advanced neoplasia (hazard ratio 3.0; 95% CI: 1.4-6.3) which remained an independent risk factor in multivariate analysis. This is in contrast to other studies showing that those with quiescent disease have a similar risk of developing CRC as those with active disease[6,43].

Post-inflammatory polyps have been associated with an increased risk of CRC. They do not in themselves have malignant potential[44]. It is possible that multiple post-inflammatory polyps increase the miss rate of dysplastic lesions or that their presence is evidence of more severe previous inflammation. A retrospective study using the Mayo Clinic centralised diagnostic index identified 188 patients with UC-associated CRC and matched them to 1528 gender and disease-extent matched controls. The presence of post-inflammatory polyps remained statistically associated with CRC even after adjusting for surveillance and anti-inflammatory treatments[45]. In the follow-up case-control study by Rutter *et al*[41], cases of CRC were significantly more likely to have post-inflammatory polyps than the controls (OR: 2.14; 95% CI: 1.24-3.70).

Gender

In their population-based study, Söderlund *et al*[30] looked specifically at the gender-related risk of IBD-CRC by identifying those diagnosed with CRC between 1960 and 2004. They compared their results with the general population using standardised incidence ratios and data obtained from national health and census registers. There were 196 cases of IBD-CRC giving an overall incidence of 110 cases per 100 000 person-years. The relative risk in males was 2.6 (95% CI: 2.2-3.1) and in females 1.9 (95% CI: 1.5-2.4) compared to the general population. The cumulative incidence at 40 years after the diagnosis of IBD was 8.3% in males and 3.5% in females.

Ekbom *et al*[29,35] found a similar relative risk for IBD-CRC in men and women whether they have UC (5.6 in men and 5.9 in women) or Crohn's disease (2.8 in men and 2.1 in women).

Family history of sporadic colorectal cancer

Patients with IBD who have a family history of sporadic CRC are at increased risk. The magnitude of the risk has been found to be a 2-3 fold increase in both case control and population-based studies. Askling *et al*[46] undertook a population-based cohort study of 19 876 patients with UC or Crohn's disease born between 1941 and 1995. They found that a family history of CRC was associated with a more than 2-fold risk of IBD-CRC (adjusted RR 2.5; 95% CI: 1.4-4.4) and those with a 1st-degree relative diagnosed with CRC before 50 years of age had a higher risk (RR 9.2; 95% CI: 3.7-23). In a retrospective cohort study, Velayos *et al*[45] also found family history of CRC to be an important risk factor for IBD-CRC in patients with UC (OR: 3.7; 95% CI: 1.0-13.2)[47].

Co-existent primary sclerosing cholangitis

Smith *et al*[43] first identified a link between UC and primary sclerosing cholangitis (PSC) in 1965. The association between Crohn's disease and PSC was identified by Atkinson and Carroll in 1964[48]. It has since been shown that the risk of IBD-CRC is greater in the presence of co-existent PSC. The adjusted relative risk for dysplasia or cancer is reported as 3.15 (95% CI: 1.37-7.27) for patients with PSC and UC as compared to those with UC alone[49]. Kornfeld *et al*[50] found the cumulative risk was 33% at 20 years and 40% at 30 years from the diagnosis of UC. Causative theories include that a high concentration of bile acids in the lumen of the colon may contribute to the increased risk[51]. Lundqvist *et al*[52] suggest that patients with PSC often have a longer duration of colitis and this in itself may explain the increased risk of CRC.

However, patients with PSC seem to have more quiescent colitis[53]. Sokol *et al*[53], also found that patients with co-existent IBD and PSC receive more 5-aminosalicylic acid (5-ASA) treatment than patients with IBD alone. Given the importance of the severity of inflammation in the aetiology of IBD-CRC this points to the existence of PSC itself having a carcinogenic effect.

Soetikno *et al*[54] conducted a meta-analysis of 11 studies to establish whether the risk of CRC is increased in patients with concomitant UC and PSC. They found that patients with UC-PSC are at increased risk of colorectal dysplasia and carcinoma compared with patients with UC alone (OR: 4.79; 95% CI: 3.58-6.41) and the risk is still increased if CRC is considered alone (OR: 4.09; 95% CI: 2.89-5.76). Broomé *et al*[55] studied 120 patients in Stockholm; 40 with UC-PSC and 80 with UC alone. They found that those with concomitant UC and PSC have a significantly higher risk of developing CRC.

CLINICAL MANAGEMENT

Chemoprevention

As with all pathology, prevention is better than cure. By focussing on the risk factors predisposing people to IBD-CRC it is hoped we can reduce the incidence of dysplasia and cancer. Any chemoprevention strategy must be acceptable to patients and physicians, in terms of safety, efficacy and cost. Ongoing colonic inflammation has been accepted as one of the causative factors behind IBD-CRC. Studies have focussed on using maintenance anti-inflammatory medications to prevent the development of dysplasia and cancer.

5-ASA compounds are used as maintenance therapy in colitis. *In vitro* studies found that they inhibit the nuclear factor kappa B pathway which is involved in tumour survival and sustaining chronic inflammation[4,56,57]. Due to the widespread use of 5-ASA compounds for maintenance therapy, prospective randomised trials are lacking with respect to chemoprevention. Velayos *et al*[58] undertook a meta-analysis of 9 studies which included a total of 1932 patients with UC. They found a protective effect of 5-ASA against colorectal cancer (OR: 0.51; 95% CI: 0.37-0.69) and colorectal cancer/dysplasia as a combined end point (OR: 0.51; 95% CI: 0.38-0.69). They did not find an association between 5-ASA use and a lower risk of dysplasia (OR: 1.18; 95% CI: 0.41-3.43) although they make the point that only 2 of the studies looked at this endpoint.

Terdiman *et al*[59] have reached a different conclusion with their observational study looking at the association between 5-ASA and IBD-CRC. They found that exposure to 5-ASA compounds during the 12 mo prior to the diagnosis of IBD-CRC was not associated with a decreased cancer risk (OR: 0.97; 95% CI: 0.77-1.23). They also found a non-statistically significant trend between a lower risk of IBD-CRC and an increased number of 5-ASA prescriptions in the previous year. Their conclusion was that 5-ASA compounds to do not have a protective effect against IBD-CRC when assessed over a short period. The important factor here seems to be the time period studied. Any preventative strategy has to be seen as a long term management approach. By using chemoprevention we are trying to prevent the genetic alterations which occur in the context of inflamed mucosa. The relationship between 5-ASA compounds and any protective effect must surely be assessed over a longer time period in order to discount it. The design of this study is too short and the results should be interpreted with caution.

The link between IBD-CRC and co-existent PSC is well established. The presence of additional bile acids in the colonic lumen is felt to contribute to this increased risk. Ursodeoxycholic acid (UDCA) decreases the amount of bile acids and studies have suggested treatment with UDCA may reduce the risk. Tung *et al*[60] found a strong relationship between UDCA and a decreased risk of colonic dysplasia (OR: 0.18; 95% CI: 0.05-0.61). The relationship remained after adjusting for gender, age at diagnosis of colitis, duration of colitis, duration of PSC, severity of liver disease, and sulfasalazine use. Pardi *et al*[61] also found a relative risk of 0.26 for colorectal dysplasia or cancer using UDCA when compared to placebo. These studies are in patients with colitis and PSC. The potential for using UDCA for patients with colitis only has not been explored. There are concerns about the side effects of using the medication in patients without PSC.

Given the potential benefit with suppression of inflammation, interest has been shown in other anti-inflammatory treatments. The role of steroids as chemopreventive agents has been explored. Evidence suggests a reduction in CRC risk with systemic and topical steroids[45,62]. However, the significant complications that occur with long-term steroid treatment make this strategy unacceptable. No chemopreventive effect has been shown with azathioprine or 6-mercaptopurine[63].

Other suggested therapies include folic acid and statins. Patients with IBD are at risk of folate deficiency. Studies have shown a protective trend against CRC but the effect is not statistically significant[64,65]. Potack *et al*[66] suggest that given that folate is safe and inexpensive, supplementation should be considered for risk reduction. A study in Israel suggested statin therapy is associated with a risk reduction in sporadic CRC and a 94% risk reduction in patients with IBD[67]. This potential benefit needs further investigation.

Surveillance

As described above, the quoted risk of developing cancer in colitis varies greatly from study to study. More recent population-based studies suggest a much lower risk than the earlier cohort studies. Methodological differences, the more recent widespread use of anti-inflammatory treatments and the advent of surveillance colonoscopy programmes may account for these differences. There is insufficient evidence to support the discontinuation

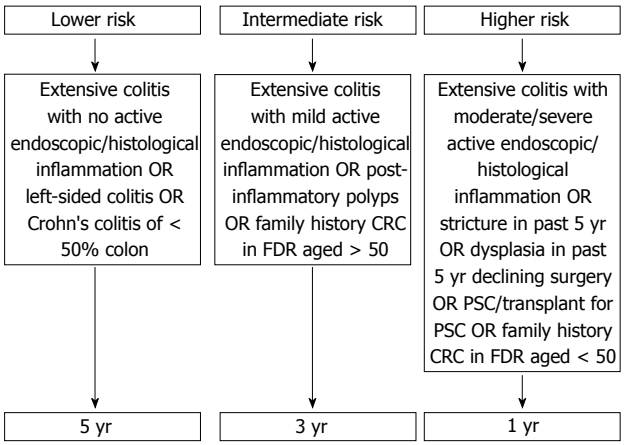

Figure 1 Surveillance recommendations for patients with colitis. OR: Odds ratio; CRC: Colorectal cancer; PSC: Primary sclerosing cholangitis; FDR: First degree relative.

of surveillance programmes and IBD is still believed to expose patients to an increased risk of CRC.

The aim of any screening or surveillance programme must be to identify early lesions to enable treatment and prevention before the development of invasive cancer. Prophylactic proctocolectomy eliminates the risk of CRC but this strategy is not acceptable to most patients or physicians. A surveillance programme must be acceptable to patients and practically possible to implement. The success of such programmes relies on patients engaging with follow up; they must understand the risks of not being tested and also that no surveillance strategy is without a miss rate.

Equally, physicians must implement the guidelines effectively. Many current guidelines advocate random quadrantic biopsies every 10 cm throughout the entire colon. This approach only visualises less than 1% of colonic mucosa. Rubin *et al*[68] found the probability of detecting dysplasia was 90% if 33 and 95% if 56 biopsies were taken. Studies have shown that clinicians do not take sufficient biopsies[69,70].

The distinct differences between sporadic CRC and IBD-CRC are important for surveillance strategies. Bowel cancer screening in the general population relies on identification of adenomatous lesions which can be resected before they transform into carcinoma. IBD-CRC poses different challenges: dysplastic lesions do not follow the adenoma-carcinoma sequence, they can be difficult to see (flat lesions), difficult to resect completely, and multifocal. A meta-analysis of 1225 patients with UC found the likelihood of finding concurrent cancer at the time of colectomy for high or low grade dysplasia was 42% and 19%, respectively[28].

Due to the increased risk, patients diagnosed with PSC who are not previously known to have IBD should have a screening colonoscopy. For patients diagnosed with UC already, yearly surveillance colonoscopy should be performed from the point of diagnosis with PSC.

There has been increased focus on targeted biopsies and methods to improve identification of dysplastic lesions. Chromoendoscopy involves spraying dye (indigo carmine or methylene blue) on to the colonic mucosa to enable more detailed examination. Rutter *et al*[71] compared consecutive, random and indigo carmine targeted biopsies. Chromoendoscopy found 7 dysplastic lesions in 157 targeted biopsies, compared to no dysplasia in 2904 non-targeted biopsies. Hurlstone *et al*[72] conducted a prospective case-controlled study of 700 patients and also found a higher yield of dysplasia using indigo carmine chromoendoscopy as compared to conventional colonoscopy with random biopsies. They found 69 dysplastic lesions using chromoendoscopy and only 24 lesions with random biopsies ($P < 0.001$). These results are supported by Kiesslich *et al*[73], who found a statistically significantly higher rate of neoplasia detection with methylene blue chromoendoscopy.

Narrow band imaging (NBI) is available on most colonoscopes. It uses optical filter technology to improve the visibility of vessels, pit pattern and other soft tissue structures. Dekker *et al*[74] performed a prospective, randomised trial to compare NBI and conventional colonoscopy. They did not find a statistically significant difference between the two methods; a similar number of dysplastic lesions were identified and missed using both methods.

Confocal laser endomicroscopy visualises the histology of the mucosa in real time. This is useful for characterising lesions rather than finding the lesion in the first place. Kiesslich *et al*[75] compared confocal chromoscopic endomicroscopy with conventional colonoscopy with random biopsies in a randomised controlled trial. They found the yield of neoplasia was increased 4.75 times using the new approach ($P = 0.005$) using 50% less biopsies. Hurlstone *et al*[76] compared confocal chromoscopic endomicroscopy to chromoendoscopy alone in a prospective, randomised controlled trial. They found that endomicroscopy increased the diagnostic yield of neoplasia 2.5 times.

However, it is already well known that there is significant inter-observer variability between even expert gastrointestinal pathologists when interpreting dysplasia. The use of confocal endomicroscopy requires the endoscopist to have the ability to interpret histological findings. For this modality to be widely used endoscopists would require extensive training to enable accuracy.

The best technique for surveillance is evolving. There is a move away from using random colonic biopsies towards targeted biopsies aimed at abnormal areas identified by newer colonoscopic techniques (chromoendoscopy, confocal microendoscopy). There has been concern regarding the specialist training needed for endoscopists to use chromoendoscopy effectively. Nevertheless, the 2010 guidelines from the British Society of Gastroenterology (BSG) recommend the use of chromoendoscopy with targeted biopsies for colitis surveillance[77].

Current guidance from the BSG advises all patients with IBD should have a screening colonoscopy approximately 10 years from symptom onset (ideally when in remission) with pancolonic dye spraying and targeted

biopsies of abnormal areas. The risk of IBD-CRC is estimated based on duration and extent of disease, co-existent risk factors (PSC, family history of sporadic CRC), and the endoscopic and histological findings at colonoscopy. The surveillance intervals are based on this assessment of risk (Figure 1)[77].

In conclusion, confirmed risk factors for IBD-CRC are duration, severity and extent of colitis, the presence of co-existent PSC and a family history of CRC. There is insufficient evidence currently to support an increased frequency of surveillance for patients diagnosed with IBD at a younger age. Evidence-based guidelines advise surveillance colonoscopy for patients with colitis 8 to 10 years after diagnosis, with the interval for further surveillance guided by risk factors (extent of disease, family history of CRC, post-inflammatory polyps, concomitant PSC, personal history of colonic dysplasia, colonic strictures)[78-80].

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ORIGINAL ARTICLE

Mutual regulation between microRNA-373 and methyl-CpG-binding domain protein 2 in hilar cholangiocarcinoma

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Abstract

AIM: To investigate the reciprocal modulation between microRNA (miRNA) and DNA methylation *via* exploring the correlation between miR-373 and methyl-CpG-binding domain protein (MBD)2.

METHODS: MiR-373 expression was examined using the TaqMan miRNA assay. Methylation of *miR-373* was investigated using methylation-specific polymerase chain reaction, and recruitment of methyl binding proteins was studied using the chromatin immunoprecipitation assay. Mutation analysis was conducted using the QuikChange™ Site-Directed Mutagenesis kit. The activity of *miR-373* gene promoter constructs and targeting at MBD2-three prime untranslated region (3'UTR) by miR-373 were evaluated by a dual-luciferase reporter gene assay.

RESULTS: In hilar cholangiocarcinoma, miR-373 decreased and was closely associated with poor cell differentiation, advanced clinical stage, and shorter survival. The promoter-associated CpG island of miR-373 gene was hypermethylated and inhibited expression of miR-373. MBD2 was up-regulated and enriched at the promoter-associated CpG island of miR-373. Methylation-mediated suppression of miR-373 required MBD2 enrichment at the promoter-associated CpG island, and miR-373 negatively regulated MBD2 expression through targeting the 3'UTR.

CONCLUSION: MiR-373 behaves as a direct transcriptional target and negative regulator of MBD2 activity through a feedback loop of CpG island methylation.

Key words: MicroRNA-373; Methyl-CpG binding domain proteins 2; Methylation; Hilar cholangiocarcinoma; Three prime untranslated region

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INTRODUCTION

Hilar cholangiocarcinoma, known as Klatskin tumor[1], is an uncommon cancer with an incidence of 0.01% to 0.2% per year[2]. Although it is relatively rare, hilar cholangio-

carcinoma displays a highly aggressive malignancy and is considered to be an incurable and rapidly lethal disease despite recent progress in diagnostic and therapeutic techniques. The 5-year overall survival after curative resection ranges from 15% to 35%, but the 10-year survival is almost zero[3]. Patients with inoperable, recurrent, or metastatic disease can only be treated with palliative therapy, such as endoscopic, percutaneous biliary drainage in combination with radiotherapy and chemotherapy. However, hilar cholangiocarcinoma is not sensitive to chemotherapy and radiotherapy, and the median survival rate of those cases is only about 5.8 mo[4].

Studies have established a direct link between aberrant DNA methylation and regulation of gene expression in human cancer[5]. Once a given sequence becomes methylated, it can directly repress transcription by either impeding recognition of transcriptional activators to DNA sequences[6], or by recruiting methyl-CpG binding domain proteins (MBPs) to modify chromatin compaction and control gene silencing[7]. The MBP family consists of five isoforms, including Mecp2, methyl-CpG-binding domain protein (MBD)1, MBD2, MBD3, and MBD4. With the exception of MBD4, which is primarily a thymine glycosylase involved in DNA repair[8], all MBPs are implicated in transcriptional repression mediated by DNA methylation. Mecp2, MBD1, and MBD2 have been demonstrated to be involved in methylation-based gene repression and also affect chromatin structure[9-11]. MBD3 lacks a functional MBD, but is an integral subunit of the histone deacetylase Mi2-NuRD complex that is recruited through MBD2[12].

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs of 18 to 24 nucleotides in length that constitute a novel class of gene regulators[13]. In general, miRNAs negatively regulate gene expression by targeting the three prime untranslated region (3'UTR), which consequently triggers mRNA degradation or translational suppression[14,15] on the basis of complementary value. miRNAs have recently been shown to play important roles in cancers, as more than 50% of miRNA genes reside in cancer-associated genomic regions, and their expression has been found to be dysregulated in various cancers[16]. Depending on the target genes, miRNAs can function as tumor suppressor genes or oncogenes[17].

Mature miRNAs are transcribed from *miRNA* genes by RNA polymerase Ⅱ. Hence, the expression of miRNAs share the same genetic and epigenetic regulation of gene function including methylation[18]. Although only subsets of miRNA genes harbor CpG islands in their promoter regions or are embedded in CpG islands, DNA methylation-mediated down-regulation of miRNAs have been reported by a number of groups[19]. Moreover, miRNA interference with DNA methylation through DNA methyltransferases (DNMTs) 3a, 3b, and DNMT1, have been observed[20-22]. These results suggest that miRNA and DNA methylation regulate one another. However, the literature has only revealed a one-way effect of miRNA on DNA methylation or miRNA modification by promoter methylation[23]. We speculate that a particular miRNA may act as a bidirectional regulator by not only impacting DNA methylation, but also *via* regulation by methylation itself.

Table 1 Relationship between miR-373 expression and clinicopathological features

Clinicopathological features	*n*	ΔC_t value of miR-373	*P* value
Age (yr)			
< 60	19	27.69 ± 3.76	0.059
⩾ 60	29	25.65 ± 4.35	
Gender			
Male	33	24.43 ± 2.43	0.877
Female	15	29.01 ± 3.76	
Tumor size (cm)			
< 2	29	25.92 ± 3.64	0.606
⩾ 2	31	25.49 ± 2.59	
Pathological type			
Adenocarcinoma	44	26.71 ± 3.18	0.390
Mucocellulare carcinoma	2	24.63 ± 3.57	
Adenosquamous carcinoma	1	23.94	
Squamous carcinoma	0		
Undifferentiated carcinoma	1	27.91	
Cell differentiation			
Well	14	19.09 ± 3.46	0.031[a]
Moderately	23	21.97 ± 1.74	
Poorly	11	28.43 ± 4.09	
Bismuth classification			
Bismuth Ⅰ	6	24.66 ± 3.31	0.082
Bismuth Ⅱ	13	27.65 ± 2.71	
Bismuth Ⅲ	17	26.99 ± 4.02	
Bismuth Ⅳ	12	26.22 ± 3.96	
Lymphatic node metastasis			
Absent	19	28.59 ± 2.53	0.224
Present	29	25.01 ± 2.64	
Clinical stages			
Ⅰ + Ⅱ	13	18.35 ± 2.62	0.017[a]
Ⅲ + Ⅳ	35	27.95 ± 3.12	

[a]$P < 0.05$ is significant.

In this study, we demonstrate that miR-373 functions as a negative regulator of MBD2 by targeting the 3'UTR. Inversely, miR-373 is restrained by MBD2 enrichment at the methylated promoter-associated CpG island. We functionally demonstrate the fact that miR-373 serves as a one directional transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

MATERIALS AND METHODS

Patients and samples

A total of 48 patients with both tumor and normal bile duct tissues, which were successfully obtained from operations conducted from January 2005 to December 2008 at Tongji Hospital in the Tongji Medical College of the Huazhong University of Science and Technology (China), were used in this study. The fresh tissues were harvested immediately after surgery, washed twice with chilled phosphate buffered saline, and immediately stored in liquid nitrogen and at -80 ℃ in our tissue bank until further use. The detailed clinical data of these patients is provided in Table 1. Written informed consent was obtained from each patient before sample collection. Ethical approval

was obtained from the Cancer Center Research Ethics Committee of Tongji Medical College and Hospital.

Cell lines and epigenetic treatment

The QBC939 cell line, originating from human common bile duct adenocarcinoma, was kindly provided by Dr. Shuguang Wang from Southwest Hospital of the Third Military Medical University (China)[24]. The HEK293 cell line was purchased from the Cell Bank of Chinese Academy of Sciences (China). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL; Carlsbad, CA, United States). For the epigenetic study, QBC939 cells were treated with 5.0 μmol 5-Aza-2-CdR for 5 d, and 200 nmol trichostatin A (TSA; Sigma-Aldrich; St. Louis, MO, United States) was added on day 5.

Taqman miRNA assay

RNA was extracted using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Carlsbad, CA, United States). cDNA synthesis and analysis of miR-373 expression were performed according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). U6 (RUN6B) was used as an endogenous control. Polymerase chain reaction (PCR) was conducted in three independent replicates for each sample. Expression of miR-373 was normalized to U6, and fold change was calculated based on the $2^{-\Delta\Delta Ct}$ method.

Genomic DNA bisulfate modification

Genomic DNA was isolated from tissues and cells using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, United States). For DNA methylation detection, 1.5 μg of genomic DNA was modified with sodium bisulfite using the EpiTect Bisulfate Kit (Qiagen). CG Genome Universal Unmethylated DNA and CG Genome Universal Methylated DNA (mDNA) (Millipore, Darmstadt, Germany) were also modified for use as positive and negative controls, respectively (100% values).

DNA methylation analysis

Methylation-specific PCR (MSP) and MethySYBR[25,26] quantative methylation-specific PCR (qMSP) were performed with primers specific for fully methylated and fully unmethylated CpG island sequences (MmiR-373, UmiR-373). Primers for converted (ActB) and unconverted (ActG) β-actin special sequences containing no CpG sites were used as a control to correct Cт values and for efficiency of bisulfite conversion, respectively. Cт values of samples MmiR-373, UmiR-373, and ActB were calculated using corresponding standard curves, after which they were corrected to DNA amount with ActB values. The sum of percent of fully methylated reference (PMR) and percent of unmethylated reference (PUR) DNA sample amounts (MmiR-373 + UmiR-373 = 100%) was calculated.

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitation (ChIP) assay was performed with the ChIP-IT™ Express kit (Active Motif, Carlsbad, CA, United States) according to the manufacturer's instructions using 2 μg ChIP-validated antibodies (Mecp2, MBD2, and mouse IgG, Active Motif; MBD1, Invitrogen, Carlsbad, CA, United States). The presence of target proteins at DNA segment were validated with primers (5'-AGATCGAGACCATCCTGGCTAACA-3'; 5'-TGAGAATGAGTCTTGCTCTGTCGC-3') to a product of 201 base pairs (bp) in size, and the enrichment of RNA polymerase Ⅱ on the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (116 bp in size) was quantified as the endogenous control. ChIP-qPCR reactions were performed using SYBR® GreenER™ Assay (Invitrogen) in a genomic DNA model. Protein enrichment was expressed as the ratio of immunoprecipitated DNA (IP-DNA) to the total amount of DNA in the chromatin sample (input). Fold change was calculated as the ratio of IP-DNA/input (target) to IP-DNA/input (RNA polymerase Ⅱ), and was then normalized to the control.

Vectors

The promoter luciferase reporter plasmid harboring the CpG island of *miR-373* gene was constructed according to a previously described approach[27] and designated pGL4-373-Prom. In brief, the *miR-373* gene locus was screened in the database (http://genome.ucsc.edu/), a 5-kb fragment upstream of the pre-miR-373 sequence was analyzed (http://www.fruitfly.org/seq_tools/promoter) to figure out the putative promoter and transcriptional start site (TSS), and a CpG island was predicted using Methprimer software (http://www.urogene.org/methprimer/). The results showed 402 bases of putative CpG islands spanning -251 to +150 bp and containing 26 CpG dinucleotides in the 5'-flank region of the human *miR-373* gene (predicted TSS is recognized as +1).

A 726 bp fragment of the *miR-373* gene (GenBank accession no. NR_029866) was amplified from QBC939 cell genomic DNA using the following PCR-specific primers: 5'-CGAT*GGTACC*TGGAAAGTGCTGCGACATTT-3' (sense), which contains an artificial *Kpn* I site, and 5'-TCAT*GCTAGC*AGAGGTTGGCCTCCAATCAT-3' (antisense), which contains an artificial *Nhe* I site and four protective bases. The PCR-amplified fragments were digested with *Kpn* I/*Hne* I and then inserted into the pGL4.22-basic plasmid (Promega, Madison,WI, United States) to generate the *miR-373* gene promoter luciferase reporter plasmid designated pGL4-373-Prom.

Precursor miR-373 clones (miRNA Accession: MI 0000781), a scrambled control clone, wild-type MBD2 3'UTR vector pEZX-MBD2-3'UTR (Gene Accession: NM_004992.3), a scrambled control, and full-length cDNA expression vector of MBD2 (pDONR™_MBD2), were purchased from GeneCopoeia company (GeneCopoeia, Rockville, MD, United States). The potential binding sequences of miR-373 on the MBD2 3'UTR were mutated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). Silencer® Select siRNA for MBD2 and the scrambled control were

purchased from Applied Biosystems (siRNA ID: s17081, Target RefSeq Number: NM_003927). The recombinants were confirmed by full-length sequencing.

siRNA knock-down study

For protein knock-down by RNAi, 5×10^6 freshly elutriated HEK293 cells were transfected with targeting MBD2-siRNA or scrambled control at a final concentration of 200 nmol, using HiPerfect Transfection Kit (Qiagen) according to the manufacturer's instructions. Seventy-two hours after transfection, the cells were harvested.

In vitro DNA methylation

pGL4-373-prom was methylated *in vitro* using M. *Sss* I (4 U/μg DNA) (New England Biolab, Ipswich, MA, United States) in the presence of 160 μmol *S*-adenosylmethionine (SAM) for 16 h. The mDNA, re-designed as pGL4-m373-prom, was digested with either *Sal* I (blocked by M. *Sss* I) or *Bam* I (not blocked by M. *Sss* I) restriction enzymes. Sensitivity to *Sal* I and resistance to *Bam* I indicated efficient DNA methylation.

Establishment of stable cell line

pGL4-m373-prom or pGL4-u373-prom were transfected into HEK293 cells in 35-mm dishes using Lipofectamine™ LTX and Plus Reagent (Invitrogen). Twenty-four hours post-transfection, cells were plated into 100-mm dishes at various densities and incubated in RPMI 1640 medium containing 2.0 μg/mL of puromycin for 7 d, followed by maintenance with 1.0 μg/mL puromycin. The stable cell line established was designated HEK-m373-prom and HEK-u373-prom.

Luciferase reporter gene assay

For promoter luciferase activity assay, pGL4-m373-prom or pGL4-u373-prom were transfected into HEK293 cells; pRL-TK was cotransfected as an endogenous control, and methylated and unmethylated pGL4-control were also used as controls. For the 3'UTR luciferase reporter assay, vectors containing WT-3'UTR or MUT-3'UTR were co-transfected with precursor miR-373 or pre-miR-neg. Cells were harvested 72 h after transfection, and luciferase activity was quantified using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, United States) according to the manufacturer's protocol.

Western blotting

Proteins (50 μg) were analyzed by Western blotting with primary antibodies against Mecp2 (1:500, Sigma-Aldrich), MBD1 (1:1000, Millipore, Darmstadt, Germany) and MBD2 (1:1000, Millipore), which produced a signal of approximately 75 kDa, 61 kDa, and 50 kDa, respectively. GAPDH (1:5000, abcam, Cambridge, MA, United States) was used as a loading control.

Statistical analysis

Data analysis was performed using SPSS for windows version 14.0 (Chicago, IL, United States. Student's test, one-way analysis of variance, and Pearson were used according to the data characteristics. Duration of hilar cholangiocarcinoma recurrence and death measured from the date of surgery was referenced against disease-free survival and overall survival time. Survival duration was calculated *via* the Kaplan-Meier method. The log-rank test was employed for comparison of cumulative survival rate and disease-free survival in the patient group. *P* values < 0.05 were considered statistically significant.

RESULTS

Down-regulation miR-373 is associated with poor cell differentiation, advanced clinical stage, and shorter survival

In patients with hilar cholangiocarcinoma, significant down-regulation of miR-373 was observed in QBC939 cells and 35 (72.92%) tumors, including seven undetectable samples ($P < 0.01$) (Figure 1A). Fold-change analysis showed a 2.94-fold decrease in the tumor group compared to the control ($P < 0.01$, Figure 1B). In regard to the correlation between miR-373 expression and clinicopathological factors, miR-373 showed low expression in specimens with poor cell differentiation ($P = 0.031$) and advanced clinical stages (stage Ⅲ, Ⅳ *vs* Ⅰ, Ⅱ) ($P = 0.017$) (Table 1), while no association was observed with age, gender, tumor size, different pathological types, Bismuth classification, or lymphatic metastasis ($P > 0.05$). Further studies were conducted to evaluate the correlation between miR-373 expression and survival. Kaplan-Meier analysis showed that down-regulation of miR-373 correlated with decreased overall survival (Figure 1C, $P < 0.05$, log-rank test) and disease-free survival (Figure 1D, $P < 0.05$, log-rank).

Promoter-associated CpG island of miR-373 is hypermethylated in hilar cholangiocarcinoma

According to the literature, the CpG island is a region of at least 200 bp, a GC percentage greater than 50%, and an observed/expected CpG ratio greater than 60%. A 402 base canonical CpG island spanning -251 to +150 bp and containing 26 CpG dinucleotides encompasses the transcription start site (TSS, recognized as +1, Figure 2A). Methylation of the promoter-associated CpG island was investigated with standard MSP and MethylSYBR. In standard MSP, methylation was present in 38 (38/48, 79.17%) tumors, including 26 homozygous and 12 heterozygous samples, which are indicated by a single methylation band or by both methylation and unmethylation bands, respectively (Figure 2B). Heterozygous methylation was also observed in five control tissues. These results were validated by qMSP and a fluorescent signal was detected in same samples. The value of PMR and PUR was 87.4% and 14.7% in the tumor and control groups, respectively (Figure 2C, $P < 0.01$).

To determine the correlation between promoter methylation and miR-373 expression, we divided methylation into four groups according to the following PMR values:

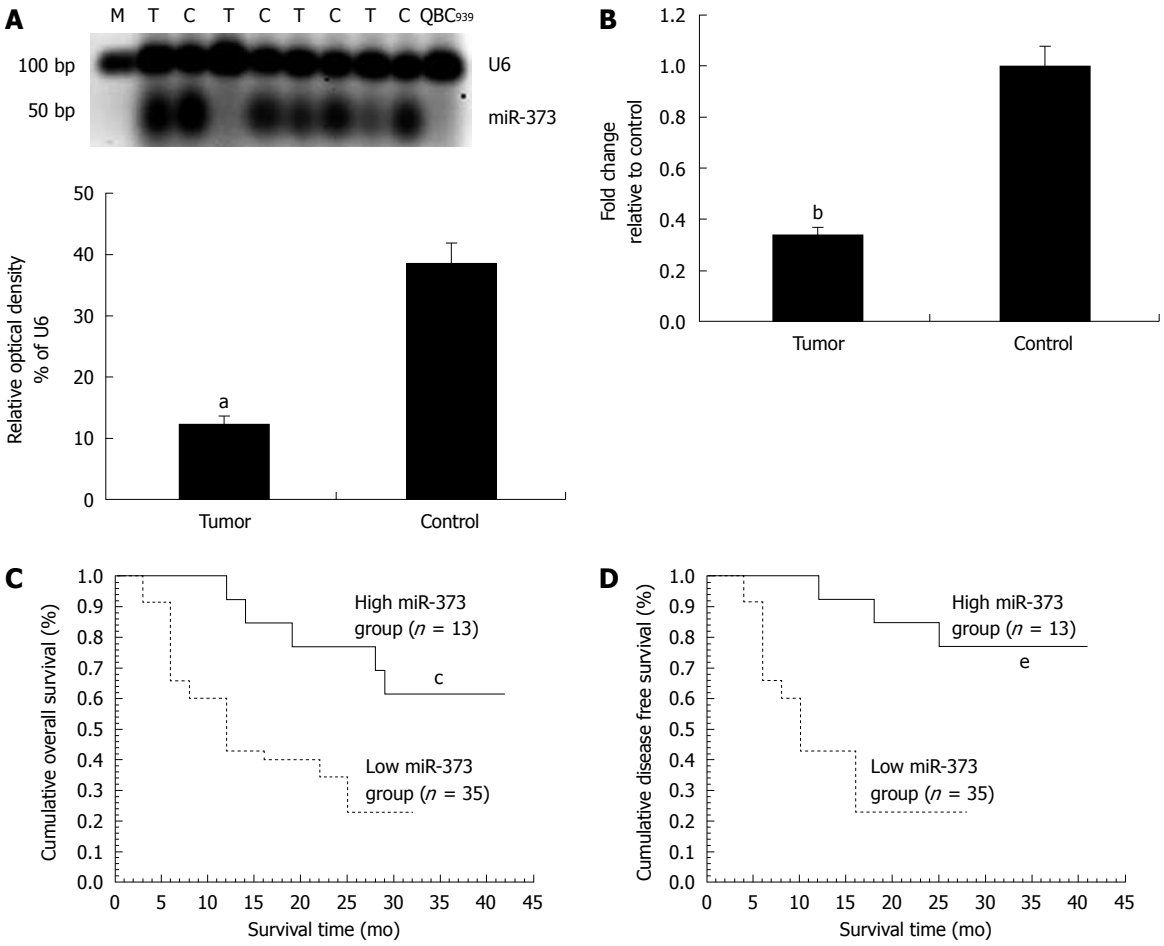

Figure 1 Expression of miR-373 and the association with clinicopathological factors in patients with hilar cholangiocarcinoma. A: Representative expression of miR-373 decreased in tumor and in QBC939 detected by RT-PCR ($^aP < 0.05$ *vs* control); B: Taqman microRNA assay of miR-373 displayed 2.94-fold down-regulation in tumor group ($^bP < 0.01$ *vs* control); C: Relationship between miR-373 expression and overall survival in the patients with hilar cholangiocarcinoma. The median overall survival time was 16.3 mo and 29.7 mo in low- and high- miR-373 group, respectively $^cP < 0.05$ *vs* high-group; D: Kaplan-Meier disease-free survival, the median disease-free survival time were 11.2 mo, 23.4 mo in low- and high- miR-373 group, respectively ($^eP < 0.05$ *vs* high-group).

(PMR above 90.00%), hypermethylation (PMR range from 42.00% to 89.99%), standard methylation (PMR range from 20.00% to 41.99%), and hypomethylation (PMR below 20.00%). Compared to its counterparts, miR-373 expression distinctly decreased in 88.5% (23/26) supermethylated samples ($P < 0.01$). There was a comparative reduction in seven hypermethylated samples ($P < 0.05$), and a dramatic increase was seen in 10 hypomethylated tumors and 43 control tissues ($P < 0.01$); no difference was detected in five standard methylated samples ($P > 0.05$). Interestingly, three supermethylated tumors were characterized by relatively high miR-373 expression (samples 14, 18, and 44). Despite these three extra tumors, promoter methylation demonstrated a inverse relationship with miR-373 expression in hilar cholangiocarcinoma (Figure 2D).

For further study on the contribution of promoter-associated CpG island methylation and inhibition of miR-373, pGL4-373-prom was constructed and methylated *in vitro* followed by transfection into HEK293 cells. As shown in Figure 3A, the relative luciferase activity in pGL4-u373-prom presented a higher level and decrease significantly in pGL4-m373-prom ($P < 0.01$).This phenomenon was also proven by data showing reactivation of miR-373 with epigenetic treatment of QBC939 cells. A 3.4-fold increase in miR-373 in cells treated with 5-Aza-2'-CdR and a 3.1-fold increase in cells treated with 5-Aza-2'-CdR and TSA were detected (Figure 3B). These results suggest that the promoter-associated CpG island acts as a cis-element of *miR-373* gene transcription, and its function can be abrogated by methylation in hilar cholangiocarcinomas and QBC939 cells.

MBP 2 is up-regulated and enriched at the promoter-associated CpG island of miR-373

Among the MeCPs, Mecp2, MBD1, and MBD2 have been established to be involved in the methylation-dependent repression of transcription. Therefore, we explored protein expression using antibodies directed against Mecp2, MBD1, and MBD2. Compared to the control, a 2.9-fold increase in MBD2 expression was found in tumors while no difference in MBD1 and Mecp2 expression were detected (Figure 4A).

The presence of Mecp2, MBD1, and MBD2 in the

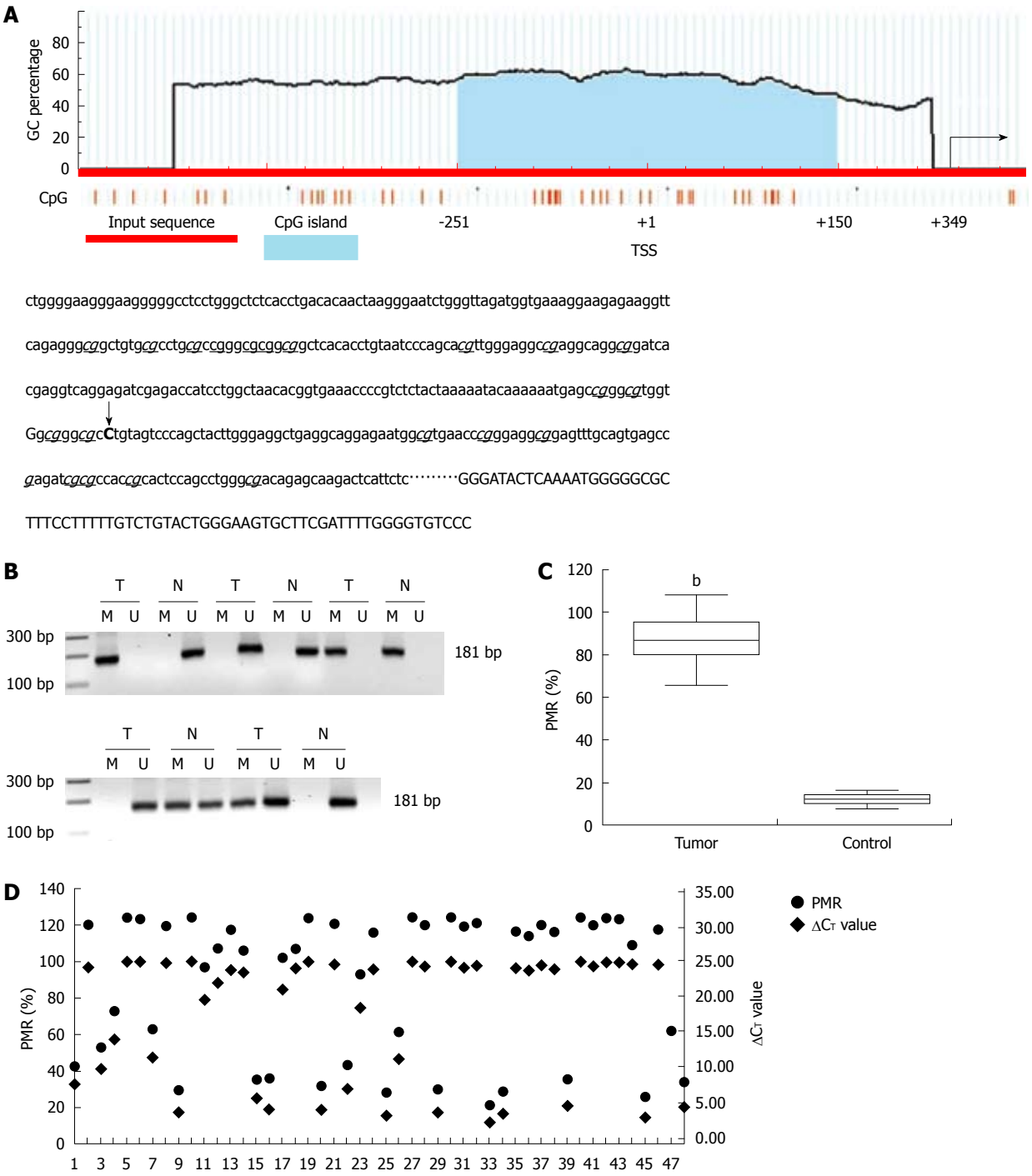

Figure 2 Methylation of miR-373 promoter-associated CpG island in hilar cholangiocarcinoma. A: Top panel, a schematic drawing of the putative CpG island in the 5'-flank region of *miR-373* gene; Bottom panel, sequence information of *miR-373* gene including partial 5'-flank region. The transcriptional start site (TSS) in boldface uppercase indicated by black arrow, 26 CpG dinucleotides are underlined and italic, pre-miR-373 sequences are capitalized; B: DNA methylation detected by methylation-specific polymerase chain reaction (MSP). Five representative cases were shown to indicate homozygous methylation (cases 1, 3), heterozygous methylation (cases 4, 5) and unmethylated (case 2), respectively. Lanes labeled M and U denote products amplified by primers recognizing methylated and unmethylated sequences; C: Percent of methylated reference (PMR) of miR-373 promoter-associated CpG island in tumor group is significant higher than control ($^bP < 0.01$ *vs* control); D: Relationship between CpG island methylation and miR-373 expression in hilar cholangiocarcinoma. A reverse correlation between PMR values and miR-373 expression could be observed (high ΔC_T value indicated low expression) excluding 3 extra samples displayed high PMR values and high mRNA expression(samples 14, 18 and 44).

region of the promoter-associated CpG island was assessed by the ChIP assay using ChIP-validated antibodies (Figure 4B). In hilar cholangiocarcinoma, the amount of CpG island fragment immunoprecipitated by MBD2 was greater than the input. ChIP-qPCR analysis showed 0.03-fold, 0.11-fold, and 0.79-fold for Mecp2, MBD1, and MBD2 compared to the endogenous control of RNA polymerase Ⅱ enrichment of GAPDH, respectively ($P < 0.01$, Figure 4B, bottom panel). These findings indicate that the fraction of promoter-associated

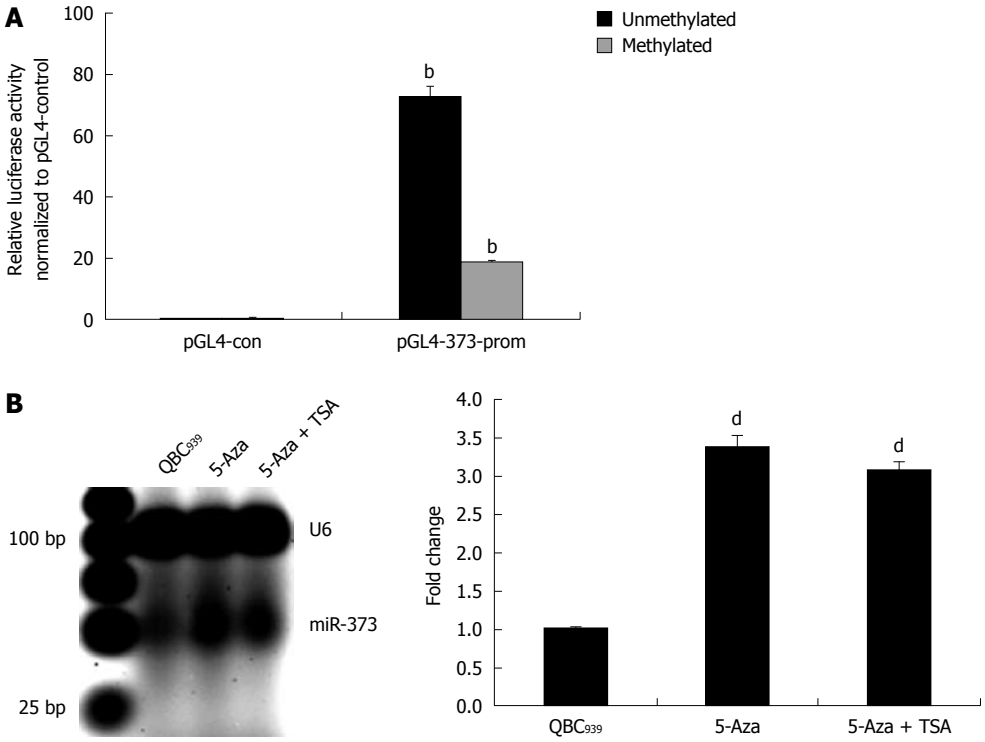

Figure 3 Methylation of CpG island regulates the expression of miR-373. A: Promoter luciferase reporter gene assay for miR-373. Prior CpG methylated resulted in dramatic decrease of luciferase activity of pGL4-m373-prom compared with pGL4-u373-prom ($^{b}P < 0.01$ *vs* control); B: miR-373 expression was reactivated by epigenetic reagents of 5'-Aza-2-CdR or combination with trichostatin A (TSA). $^{d}P < 0.01$ *vs* untreated QBC939.

CpG island is selectively immunoprecipitated by MBD2, but not by Mecp2 and MBD1. In addition, fold enrichment analysis showed a remarkable correlation between miR-373 promoter methylation and MBD2 enrichment in four groups with different frequencies of methylation ($P < 0.01$, Figure 4C).

Methylation-mediated suppression of miR-373 requires MBP 2 enrichment at promoter-associated CpG island

In QBC939 cells and hilar cholangiocarcinoma, we evaluated whether inhibition of miR-373 was closely related to hypermethylation and enrichment of MBD2 at the promoter-associated CpG island. Specifically, to unravel whether the enrichment of MBD2 is critical for inactivation of miR-373, exogenous MBD2 expression was induced in stable cell lines of HEK-u373-prom or HEK-m373-prom. The ChIP assay was performed 72 h post-transfection, and fold change analysis showed a 6.2-fold enrichment of MBD2 in pGL4-m373-prom compared to wild-type HEK293 cells, while no change was found in the pGL4-u373-prom group (Figure 5A, $P < 0.01$).

Further knock-down studies in QBC939 were performed to reduce endogenous MBD2 by a specific siRNA. A 4.3-fold increase in miR-373 expression was observed in knock-down cells compared to wild-type QBC939 cells (Figure 5C). Sequential ChIP assay revealed that knock-down of MBD2 resulted in depletion of MBD2 enrichment at the CpG island (Figure 5B). Furthermore, knock-down of MBD2 was not compensated by the binding of MBD1 or Mecp2. In addition, in MBD2 knock-down QBC939 cells, 5-Aza-2-CdR and TSA treatments had an innocent effect on MBD2 enrichment (Figure 5D). These findings suggest that the enrichment of MBD2 is specific to the methylated region of miR-373 promoter-associated CpG island, MBD2 likely mediates CpG methylation-dependent inhibition of miR-373 in hilar cholangiocarcinomas.

miR-373 negatively regulates MBPs 2 expression by targeting the 3'UTR

One putative miR-373 binding site was predicted to have greater specificity to MBD2 3'UTR, ranging from dinucleotide 295 to 301 bp, as predicted by four algorithms (TargetScan, PicTar, miRanda, miRbase Target) (Figure 6A). To investigate whether the 3'UTR of MBD2 is a functional target of miR-373, wild-type MBD2-3' UTR vector was transfected into HEK293 cells with pre-miR-373, which led to a decrease of 55.8% reporter activity compared to the pre-miR-neg (Figure 6B, $P < 0.001$). After the conserved targeting regions for miR-373 recognition were mutated, relative luciferase activity of the reporter gene was also restored (Figure 6B). These observations suggest that the predicted complementary sequence in MBD 3'UTR is a functional element of miR-373.

On the contrary, enhanced expression of miR-373 by transfecting pre-miR-373 into QBC939 cells resulted in a significant reduction of MBD2 protein (Figure 6C). Furthermore, reactivation of miR-373 expression in epigenetic-treated QBC939 cells (Figure 3B) led to an inhibition

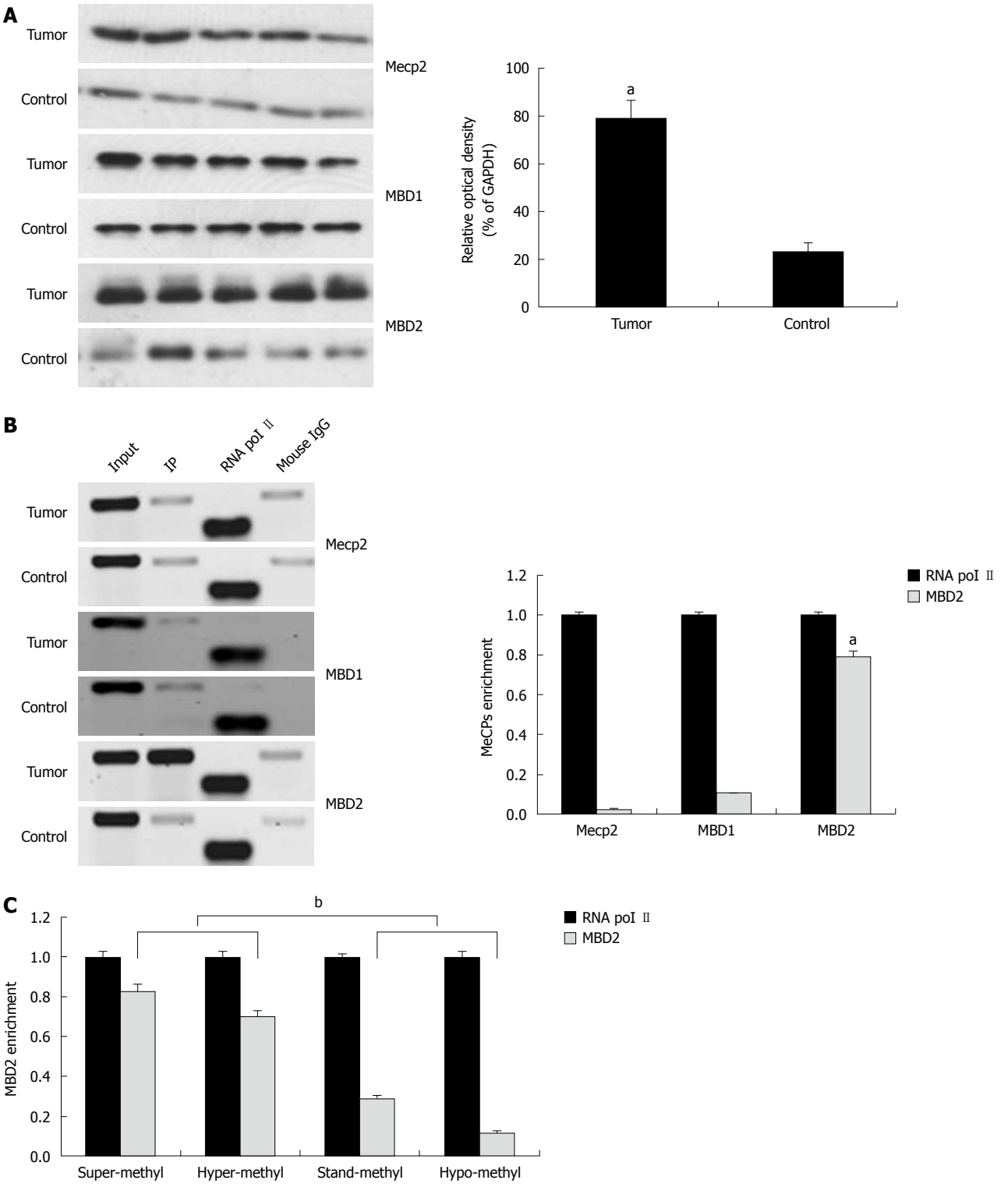

Figure 4 Methyl-CpG binding domain proteins expression and enrichment in fragment of promoter-associated CpG island. A: Expression methyl-CpG binding domain proteins (MBPs) in hilar cholangiocarcinoma. Compared to control, 2.9-fold increase of methyl-CpG-binding domain protein (MBD)2 was found while no difference of MBD1 and Mecp2 were detected; B: Chromatin immunoprecipitation (ChIP)-polymerase chain reaction analysis showed selective enrichment of MBD2 at region of CpG island; C: Correlation between MBD2 enrichment and different frequency of CpG island methylation. Remarkable difference was observed between super-/hyper-methylation and stand-/hypo-methylation group ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

of MBD2 protein (Figure 6D). Taken together, these findings suggest that MBD2 3'UTR carries a miR-373 regulatory site, and miR-373 can negatively regulate MBD2 through binding to the miRNA locus of MBD2 3' UTR.

DISCUSSION

In previous studies, miR-373 has displayed controversial characteristics in different cancers. In testicular germ cell tumors[27], esophageal cancer[28], and breast cancer[29],

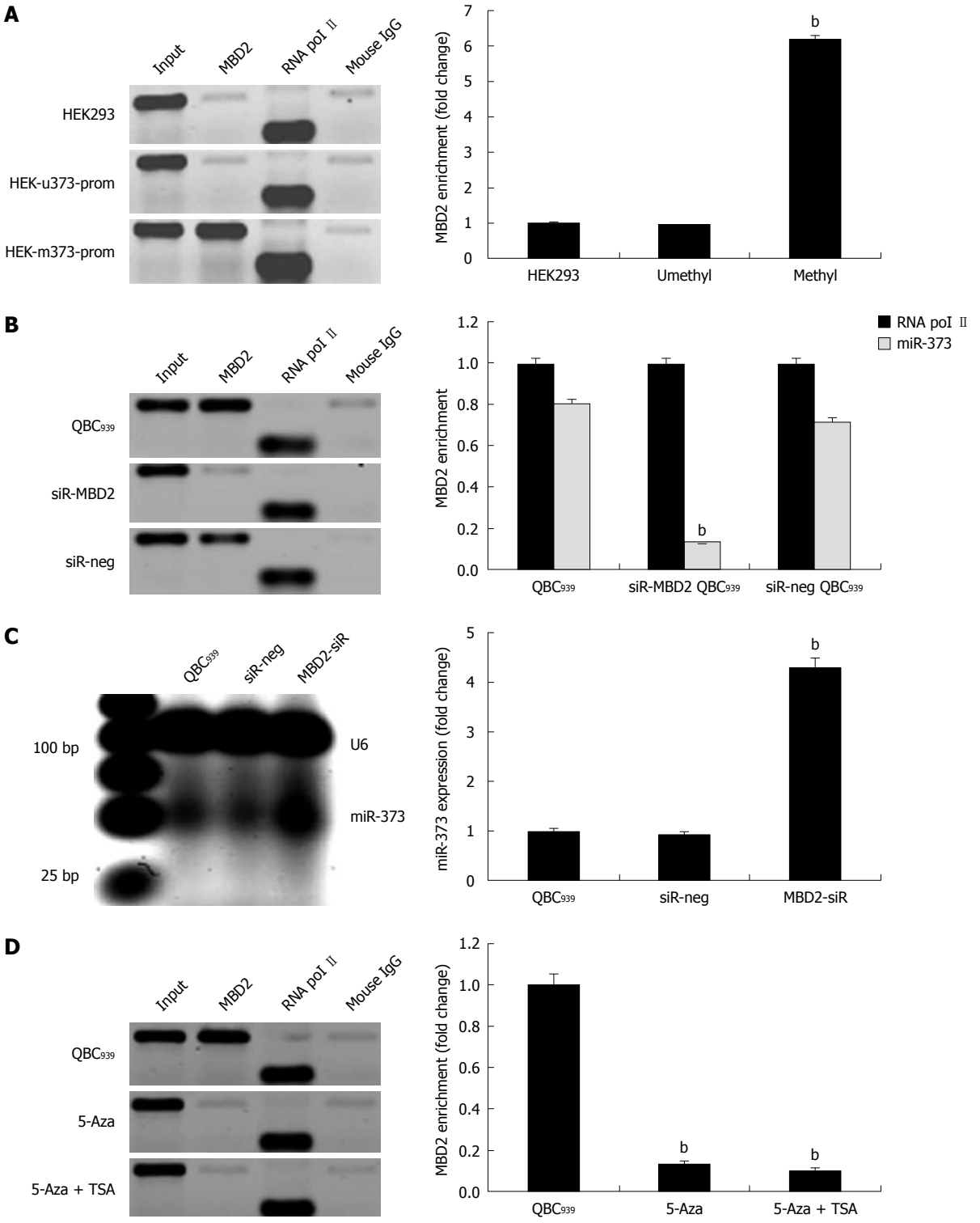

Figure 5 Enrichment of methyl-CpG-binding domain protein 2 at CpG island region is essential for methylation-mediated silencing of *miR-373* gene. A: Enrichment of methyl-CpG-binding domain protein (MBD)2 in fragment of CpG island in HEK-m373-prom stable cells showed 6.2-fold increase compared to control and no significant change was detected in HEK-u373-prom cells; B: MBD2 enrichment in MBD2-siRNA QBC939 cells presented dramatically deduction compared to QBC939 cells; C: Knock-down of MBD2 induced a increase of miR-373 expression in QBC939 cells; D: Epigenetic treatment of QBC939 cell with 5-Aza-CdR or combination with trichostatin A (TSA) eliminated the recruitment MBD2 at the region of miR-373 promoter-associated CpG island. $^{b}P < 0.01$ *vs* background.

miR-373 behaves as a novel oncogene. Whereas in prostate cancer[30] and malignant cholangiocytes, including the extrahepatic cholangiocarcinoma cell line[31], miR-373 shows characteristics of a tumor suppressor. Regardless of this divergence, it has been well established that miR-373 participates in tumorigenesis, invasion, and metastasis by mediating gene expression.

In this study, we show that miR-373 is dramatically down-regulated in hilar cholangiocarcinoma, and correlates closely with poor cell differentiation, advanced

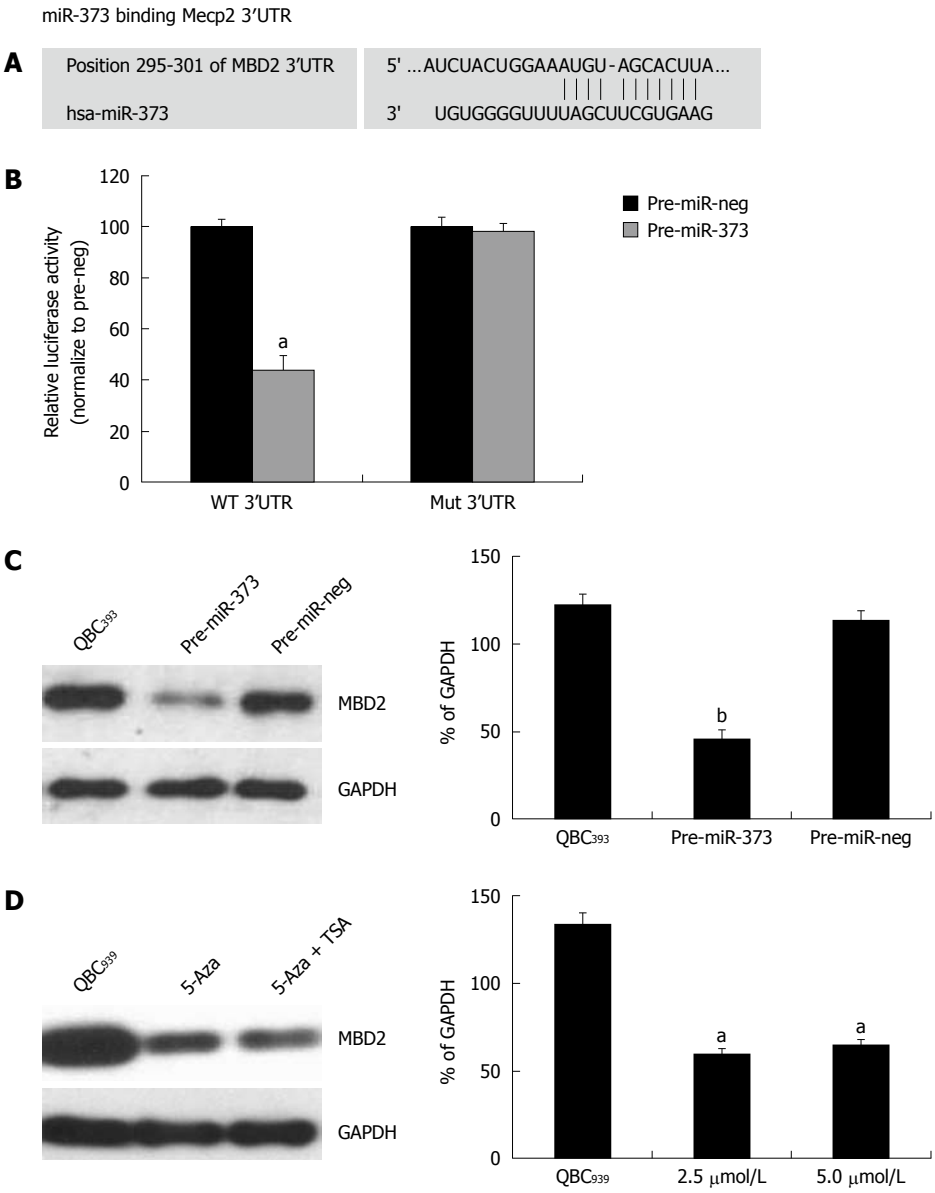

Figure 6 miR-373 negatively regulates methyl-CpG-binding domain protein expression through binding to three prime untranslated region. A: miRNA target prediction screened one computative miR-373 binding site at methyl-CpG-binding domain protein (MBD)2-three prime untranslated region (3'UTR); B: 3'UTR luciferase reporter assay showed a reduction of relative luciferase activity of wild-type MBD2 3'UTR by pre-miR-373 in HEK293 cells; C: Exogenous miR-373 down-regulates MBD2 protein in QBC939; D: Epigenetic treatment of QBC939 cells inhibites MBD2 protein following reactivation of miR-373 (Figure 3A). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* background. TSA: Trichostatin A; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

clinical stages, shorter overall survival, and disease-free survival. Our findings are in agreement with the last two reports. Although it is difficult to definitively explain these directly opposing results, the expression pattern of individual miRNAs with strict tissue- and clinical-feature-specificity[32], and the different target genes involved in the unique regulation network of various cancers, could lead to these discrepancies.

Epigenetic dysregulation of miRNAs in human cancer constitutes an emerging mechanism implicated in the development of cancer[33]. Great effort has been devoted to understanding the relevance of aberrant CpG methylation patterns, and their roles in gene transcription. The inverse correlation between miR-373 expression and hilar cholangiocarcinoma progression prompted us to study the molecular mechanisms underlying *miR-373* gene inhibition. In the present study, miR-373 promoter-associated CpG island was found to be hypermethylated in tumor tissues and QBC939 cells. Reciprocal assays were performed with demethylation of the CpG island by treatment of QBC939 cells with 5-aza-CdR in the absence or presence of combination with TSA, which contributes to the reactivation of miR-373. In addition, pre-methylation of pGL4-373-prom *in vitro* inhibited luciferase activity. Together, the results presented here provide evidence that promoter-associated CpG island methylation is a major cause of *miR-373* gene suppression in hilar cholangiocarcinoma.

Promoter-associated CpG-methylation, along with MBPs and HDACs, has been identified as a major epi-

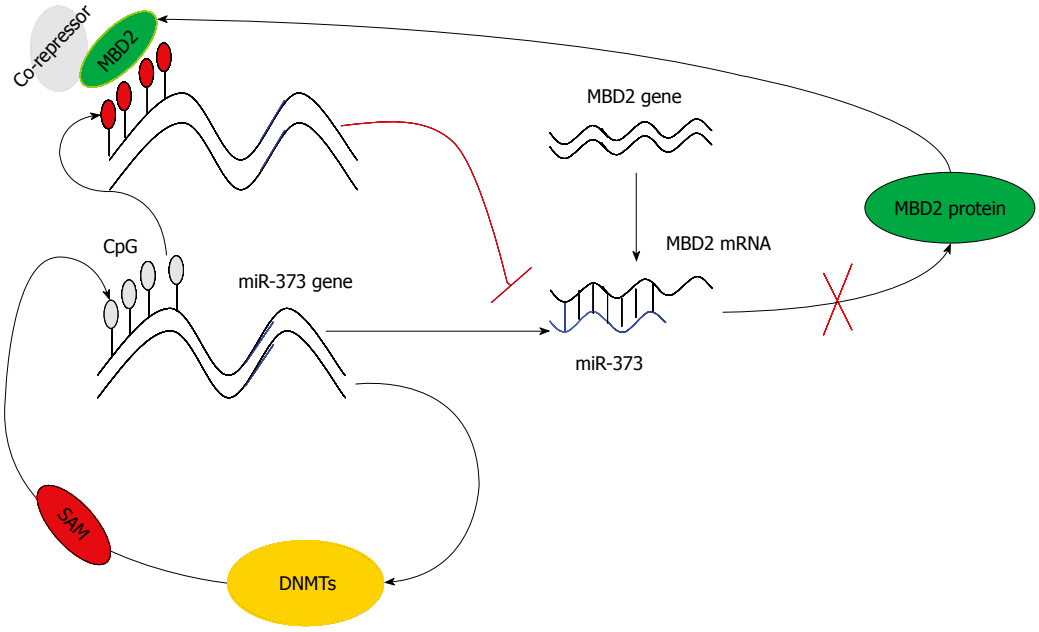

Figure 7 Dual regulation between miR-373 and methyl-CpG-binding domain protein 2. miR-373 is one direct transcriptional target and negative regulator to methyl-CpG-binding domain protein (MBD)2 through a feedback loop of CpG methylation. SAM: S-adenosylmethionine; DNMTs: DNA methyltransferases.

genetic event in the loss of gene expression during tumor progression[34]. In this study, we further investigated the involvement of MBPs in methylation-mediated suppression of miR-373 in hilar cholangiocarcinoma. MBD2 is an exclusive MBP that is aberrantly expressed in hilar cholangiocarcinoma. ChIP assays showed that MBD2 selectively bound to methylated CpG sequences. Moreover, silencing conferred by DNA methylation and MBD2 enrichment in QBC939 cells was reversed by treatment with 5-aza-CdR and TSA. These findings indicate that MBD2 plays an important role in recruiting transcription-repressive machinery to the methylated promoter, thereby suppressing transcriptional activation of miR-373. Confirming these findings, siRNA knock-down of MBD2 triggered a stimulation of miR-373 in QBC939 cells. Taken together, these data suggest that MBD2 is an important factor in methylation-mediated inhibition of miR-373.

The reciprocal correlation of expression between miR-373 and MBD2 encouraged us to explore whether miR-373 is a negative regulator of MBD2. To this end, four algorithms were used to predict the alignment of miR-373 with MBD2 3'UTR. In consensus, the seed region of miR-373 matched nucleotides 295-301 of the MBD2 3'UTR, suggesting the ability of miR-373 to directly bind to MBD2 mRNA. However, not all miRNAs identified in this manner are likely to be functional since factors such as steric hindrance may render them inaccessible to the mRNA[35]. Hence, functional validation experiments including transfection of miR-373 precursors showed that miR-373 can down-regulate the relative luciferase activity of MBD 3'UTR reporterer vectors and MBD2 protein in QBC939 cell lines. These findings indicate that miR-373 functionally regulates the expression of MBD2 by targeting the 3'UTR.

In this study, several interesting observations were made. First, the heterozygous methylation of miR-373, as indicated by both methylated and unmethylated bands in MSP, was detected in 12 cancers and five normal bile duct tissues. This is contrary to the 'all-or-none' manner of DNA methylation in regulating gene expression. Although the mechanism is unclear, there are several points to keep in mind: (1) *miR-373* gene exhibits allele-specific DNA methylation (ASM) which means that only one allele is methylated and the other one is unmethylated[36]. ASM has been documented in a number of cancer cases except imprinted regions and X chromosomes; (2) samples contain normal and malignant cells although multiple efforts have been adapted to obtain purified tissues; and (3) miR-373 displays discrepant methylation in various differentiated cells. Secondly, in primary samples, three supermethylated tumors were characterized with relatively high miR-373 expression. The relevant mechanism underlying transcription that escapes methylation-mediated suppression is unknown, but whether MBPs bind effectively to the methylated CpG dinucleotides may determine the expression level.

Based on these findings, we conclude that due to the hypermethylation of the promoter-associated CpG island and enrichment of MBD2, the function of miR-373 is restrained rendering it unable to inhibit MBD2. As a result, the expression of MBD2 is predominantly enhanced, leading to a strong inhibitory effect on miR-373 (Figure 7). This dysregulation ultimately results in the promotion of tumorigenesis and the development of hilar cholangiocarcinoma. In conclusion, our study proves that miRNA-373 behaves as a direct transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

COMMENTS

Background

Both DNA methylation and microRNAs (miRNAs) are epigenetic and play vital roles in tumorigenesis and development of human malignance. DNA methylation represses transcription by impeding recognition of transcriptional activators to DNA sequences or recruiting methyl-CpG binding domain proteins (MBPs) to modify chromatin compaction and control gene silencing. miRNAs regulate gene expression mainly by binding to the three prime untranslated region of target mRNAs, leading to mRNA degradation or translation inhibition. Many studies have reported that the expression of miRNAs gene is regulated by DNA methylation, and in addition, DNA methyltransferases and MBPs are regulated by miRNAs.

Research frontiers

Hilar cholangiocarcinoma displays highly aggressive malignancy. Many studies have reported miRNA expression and DNA methylation in hilar cholangiocarcinoma. In this study, the authors report evidence of the role of miR-373 in hilar cholangiocarcinoma. In particular, they show that miR-373 behaves as a direct transcriptional target and negative regulator of methyl-CpG-binding domain protein (MBD)2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma.

Innovations and breakthroughs

It has been well established that miR-373 participates in tumorigenesis, invasion, and metastasis by mediating gene expression. In this study, the authors demonstrate that miR-373 is dramatically down-regulated in hilar cholangiocarcinoma, and closely correlates with poor cell differentiation, advanced clinical stage, shorter overall survival, and disease-free survival. The authors show that due to the hypermethylation of the promoter-associated CpG island and enrichment of MBD2, function of miR-373 is restrained, rendering it unable to inhibit MBD2. As a result, MBD2 expression is predominantly enhanced and has a strong inhibitory effect on miR-373. This dysregulation finally promotes the tumorigenesis and development of hilar cholangiocarcinoma.

Applications

This study provides the first evidence showing that miR-373 behaves as a direct transcriptional target and negative regulator of MBD2 through a feedback loop of CpG methylation in hilar cholangiocarcinoma. These results shed light on the mutual regulation between miRNA-373 and MBD2, which may eventually serve as useful biomarkers as well as therapeutic targets.

Peer review

This study demonstrates the role of miR-373 in cholangiocarcinoma. In particular, the authors showed that miR-373 acts through a feedback loop of CpG methylation. This study is well designed and performed, and is of great interest for its novelty and impact in the field.

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BRIEF ARTICLE

Moro orange juice prevents fatty liver in mice

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Abstract

AIM: To establish if the juice of *Moro*, an anthocyanin-rich orange, may improve liver damage in mice with diet-induced obesity.

METHODS: Eight-week-old mice were fed a high-fat diet (HFD) and were administrated water or *Moro* juice for 12 wk. Liver morphology, gene expression of lipid transcription factors, and metabolic enzymes were assessed.

RESULTS: Mice fed HFD displayed increased body weight, insulin resistance and dyslipidemia. *Moro* juice administration limited body weight gain, enhanced insulin sensitivity, and decreased serum triglycerides and total cholesterol. Mice fed HFD showed liver steatosis associated with ballooning. Dietary *Moro* juice markedly improved liver steatosis by inducing the expression of peroxisome proliferator-activated receptor-α and its target gene *acylCoA-oxidase*, a key enzyme of lipid oxidation. Consistently, *Moro* juice consumption suppressed the expression of liver X receptor-α and its target gene fatty acid synthase, and restored liver glycerol-3-phosphate acyltransferase 1 activity.

CONCLUSION: *Moro* juice counteracts liver steatogenesis in mice with diet-induced obesity and thus may represent a promising dietary option for the prevention of fatty liver.

Key words: Liver steatosis; Anthocyanins; Lipogenesis; Lipid oxidation

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic

metabolic disorder with significant impact on cardiovascular and liver-related mortality[1,2]. NAFLD is closely associated with obesity, dyslipidemia, diabetes and the full spectrum of the metabolic syndrome[3,4], with insulin resistance as a common pathophysiological determinant[5]. Lifestyle, that is, dietary habits and physical activity, plays a pivot role in the pathogenesis of the metabolic syndrome[6]. Likewise, dietary factors have been shown to exert a major role in the development of fatty liver[7]. Recently, the consumption of foods and beverages containing fructose has been identified as a risk factor for NAFLD[8]. Nonetheless, despite their fructose content, fruits are also rich in different polyphenolic compounds that exert several beneficial effects on health, mainly by modulating expression of key enzymes involved in glucose sensitivity and lipid homeostasis[9].

Anthocyanins (ACNs) are water-soluble plant polyphenolic pigments that confer a typical blue, red or purple color, and are contained especially in berries, blood oranges and pigmented corn and potato[10]. ACNs have been attributed several putative therapeutic roles, including beneficial effects on obesity and related metabolic complications[11]. In this respect, Tsuda *et al*[12] firstly showed that ACN from purple corn prevented obesity, dyslipidemia and visceral fat inflammation in mice fed a high-fat diet (HFD). Recently, a further demonstration of the putative hepatoprotective properties of ACN has been provided by Liu *et al*[13], who observed that administration of protocatechuic acid, the major *in vivo* metabolite of the main ACN cyanidin-3-*O*-β-glucoside, reduced gene expression of lipogenic enzymes in the liver, thus leading to a decreased hepatic lipid accumulation. *In vitro* and *in vivo* experiments suggest that ACNs can modulate gene expression of several adipocytokines and regulate the pathways involved in lipogenesis and fat accumulation[12,14]. ACNs are contained also in blood oranges, a variety of sweet orange (*Citrus sinensis*) with an intense red pigmentation[15]. A recent study has demonstrated that blood orange consumption inhibits fat accumulation in mice[16] and may represent a promising dietary tool for the treatment of obesity.

In the current study, we aimed at clarifying whether consumption of the juice of *Moro*, a blood orange cultivated in Sicily, Italy, improved liver steatosis in mice fed HFD; a physiological model of NAFLD and metabolic syndrome.

MATERIALS AND METHODS

Animals and treatments

All procedures fulfilled the Italian Guidelines for the Use and Care of Laboratory Animals. Eight-week-old male C57BL6/J mice were purchased from Charles River Laboratories (Calco, Italy). Animals were maintained in a temperature- and light-controlled facility for 12 wk. Diets were obtained by Harlan Teklad (Madison, WI, United States). The standard diet (SD) provided 3.3 kcal/g with 60% carbohydrates, 23% proteins and 17% fat. The HFD provided 5.2 kcal/g with 60% fat, 20% proteins and 20% carbohydrates. Mice were distributed in three groups: group Ⅰ included six mice fed SD and permitted *ad libitum* consumption of water (SD + water); group Ⅱ comprised six mice fed HFD and permitted *ad libitum* consumption of water (HFD + water); group Ⅲ comprised six mice fed HFD and permitted *ad libitum* consumption of *Moro* orange juice instead of water (HFD + *Moro*). *Moro* fruits were collected in the experimental farm of the Research Center for Citric Culture and Mediterranean Crops (Acireale, Italy). Fruits were immediately stored at 4 ℃ and squeezed a few days later; the juice obtained was pre-filtered and stored at -20 ℃ in aliquots of 0.5 L. Every 2 d, frozen juice aliquots were thawed, filtered and put in the bottle of each cage. Fruit juice analysis was performed as previously described[16]. Food and beverage consumption was recorded twice weekly; body weight was recorded weekly. After sacrifice by CO_2 asphyxiation, blood and liver samples were obtained, processed and stored for further analysis.

Histopathology

Formalin-fixed paraffin-embedded liver sections were stained with haematoxylin-eosin and Masson's trichrome, using standard procedures. Liver injury was blindly evaluated according to the NAFLD activity score.

Biochemical analysis

Serum glucose, total cholesterol, triglycerides and alanine aminotransferase (ALT) were measured using Reflotron Plus system from Roche Diagnostic (Milan, Italy). Liver triglycerides content was measured using a serum/tissue triglyceride colorimetric kit (Biovision, Mountain View, CA, United States). Insulin tolerance test (ITT) was performed on 5-h starved mice, and glycemia was measured immediately before and 15, 30 and 60 min after intraperitoneal injection of 0.4 U/kg recombinant human insulin. The total amount of ACN in *Moro* juice was determined as previously described[16]. Glycerol-3-phosphate acyltransferase 1 (GPAT1) activity was assayed as previously described [17].

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted by homogenizing snap frozen liver samples in TRIzol reagent (Invitrogen, Milan, Italy). Quantitative real-time polymerase chain reaction (PCR) was performed in 7900HT Fast Real-Time PCR System Applied Biosystems (Applied Biosystems, Foster City, CA, United States), using the EXPRESS SYBR GreenER™ qPCR SuperMix with Premixed ROX (Invitrogen).

Reactions were performed in a 20 μL mixture containing cDNA, specific primers of each gene, and the SYBRR GreenER™ qPCR SuperMix. The specific PCR products were detected by the fluorescence of SYBR Green, the double-stranded DNA binding dye. The relative mRNA expression level was calculated by the threshold cycle (Ct) value of each PCR product and normalized with that of glyceraldehyde-3-phosphate de-

Table 1 Primer sequences for real-time polymerase chain reaction

Gene name	Forward	Reverse
PPAR-α	5′-AGTCAAGGTGTGGCCCAAGGT-3′	5′- TGTCTATCGGACACTAGCGGAGGC-3′
AOX	5′-CTTGTTCGCGCAAGTGAGG-3′	5′-CAGGATCCGACTGTTTACC-3′
LXR-α	5′-TGCCATCAGCATCTTCTCTG-3′	5′-GGCTCACCAGCTTCATTAGC-3′
FAS	5′-AGCCCACGTCGTAGCAAACCA-3′	5′-GCAGGGGCTCTTGACGGCAG-3′
HMG-CoA reductase	5′-CCTGACACTGAACTGAAGCG-3′	5′-TCTTTCCAG AACACAGCACG-3′
GAPDH	5'-ACCACCATGGAGAAGGCCGG-3'	5'-CTCAGTGTAGCCCAAGATGC-3'

PPAR: Peroxisome proliferator-activated receptor; AOX: Acyl-CoA oxidase; LXR: Liver X receptor; FAS: Fatty acid synthase; HMG: Hydroxy methylglutaryl; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase .

hydrogenase by using comparative $2^{\Delta\Delta Ct}$ method. Primer sequences are shown in Table 1.

Statistical analysis

Statistical analysis was performed by GraphPad Prism software (San Diego, CA, United States). Data are the results of three independent experiments. All results were expressed as mean ± SE. One-way analysis of variance with Bonferroni post-hoc analysis was used for parametric data. Kruskal-Wallis test was used for non-parametric data. P values < 0.05 were considered significant.

RESULTS

Moro juice improves dyslipidemia and enhances insulin sensitivity

Moro juice had an ACN content of 85 mg/L. Food intake was identical among the three experimental groups (SD + water, 3.1 ± 0.5 g/d *vs* HFD + water, 3.0 ± 0.8 g/d *vs* HFD + *Moro*, 3.1 ± 0.7 g/d). The amount of *Moro* juice intake (4.1 ± 0.75 mL/d) did not differ from the amount of water intake in the control groups (4.0 ± 1.2 mL/d). All mice had similar body weight at the start of the experiment, and after 12 wk, mice fed HFD + water had higher body weight compared with SD mice (Figure 1A). Strikingly, mice fed HFD + *Moro* had the same body weight gain as mice fed SD. This effect on body weight gain occurred despite the fact that mice fed HFD + *Moro* received a 10% higher energy intake, due to the sugar content of the juice, as compared to mice fed HFD + water (Figure 1A). Obese mice had increased serum total cholesterol, triglycerides and ALT as compared to the SD group (Figure 1B-D). *Moro* juice decreased serum total cholesterol and triglycerides, and reduced serum ALT to the levels of lean controls (Figure 1B-D). Furthermore, orange juice consumption significantly enhanced insulin sensitivity, as demonstrated by the area under curve derived from ITT, which was 8685 ± 516 in mice fed HFD + water and was reduced to 7225 ± 718 in the HFD + *Moro* group (Figure 2).

Moro juice induces liver peroxisome proliferator-activated receptor-α and inhibits liver lipogenesis

Liver sections of mice fed HFD + water showed moderate steatosis with a panacinar pattern (Figure 3B), mild lobular inflammation, and diffuse hepatocyte ballooning. In the HFD + *Moro* group, steatosis was almost absent (Figure 3C). Consistently, lobular inflammation and ballooning degeneration was less pronounced throughout the hepatic parenchyma. Fibrosis, assessed by Masson's trichrome, was not found in any mice after 12 wk HFD (data not shown). Biochemical analysis confirmed that *Moro* juice induced a marked decrease of liver triglyceride content in mice fed HFD (Figure 3D).

HFD was associated with impaired expression of key transcription factors and metabolic enzymes involved in lipid homeostasis. In particular, we found a decrease in the gene expression of peroxisome proliferator-activated receptor (PPAR)-α (Figure 4A) and acyl-CoA oxidase (AOX) (Figure 4B) and a significant increase in the expression of liver X receptor (LXR)-α (Figure 4C), fatty acid synthase (FAS) (Figure 4D) and hydroxy methylglutaryl (HMG)-CoA reductase (Figure 4E). *Moro* juice markedly decreased the mRNA levels of LXR-α, FAS and HMG-CoA reductase but augmented the mRNA levels of PPAR-α and AOX (Figure 4A-E). Consistent with gene expression findings, GPAT1 activity was restored to the levels of lean animals by *Moro* juice consumption (Figure 4F).

DISCUSSION

In this study, we explored the effect of the consumption of the juice of *Moro*, an ACN-rich orange cultivated in the Mediterranean region, on liver steatosis in mice with diet-induced obesity. In previous experiments in mice fed SD, we observed that C57BL6 mice and other strains tolerated the substitution of drinking water with orange juice as the only drinking source, had similar food intake, and did not show any behavioral abnormality. Here, we demonstrated that *Moro* juice drinking reversed most of the metabolic abnormalities exhibited by obese mice, including fatty liver.

Our results are in agreement with previous experimental data suggesting that ACNs from different fruits and vegetables are able to exert beneficial effects on several metabolic aspects related to obesity[11]. One common effect of ACN administration both in diet-induced and genetic models of obesity is the reduction of body weight and of visceral fat. In this respect, Kwon *et al*[18] showed that ACN extracted from black soybean led to improvement in dyslipidemia and a decrease in visceral

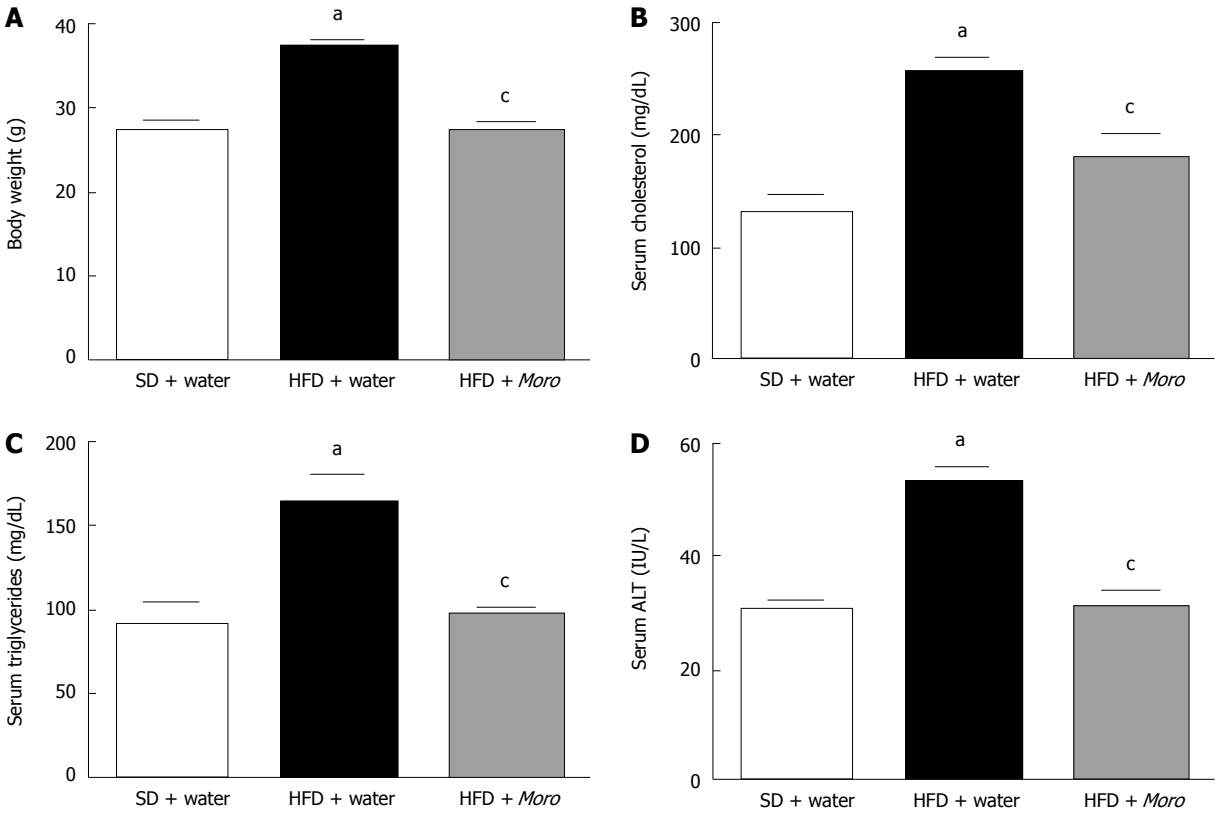

Figure 1 Effects of *Moro* juice on body weight and serum parameters. A: Body weight was markedly increased in mice fed high-fat diet (HFD) and was decreased to the levels of lean mice by *Moro* juice; B: Serum total cholesterol was significantly reduced in mice drinking *Moro*; C and D: Serum triglycerides (C) and alanine aminotransferase (ALT) (D) were restored to the levels of lean mice. [a]$P < 0.05$ *vs* standard diet (SD) + water; [c]$P < 0.05$ *vs* HFD + water.

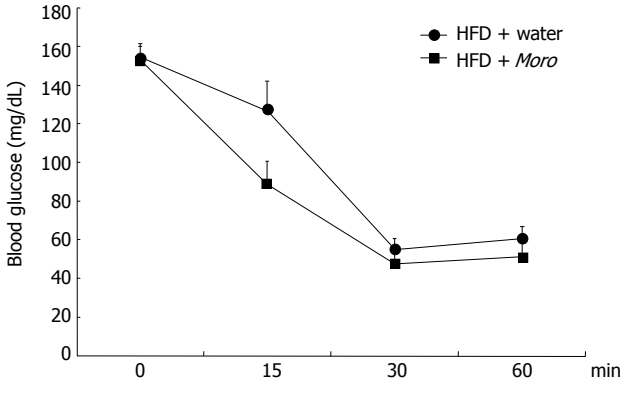

Figure 2 Effects of *Moro* juice on insulin sensitivity. Insulin tolerance test was performed with 0.4 U/kg recombinant human insulin in the two groups of mice fed high-fat diet (HFD); area under curve of blood glucose was 8685 ± 516 in mice fed HFD + water and was reduced to 7225 ± 718 in HFD + *Moro* ($P < 0.05$).

adiposity in rats fed HFD. Similarly, extracts from tart cherries ameliorate dyslipidemia and decrease liver fat content in genetically insulin-resistant rats[19], as does cyanidin 3-glucoside in db/db mice[20].

As regards the molecular events underlying the effects of *Moro* on liver lipid metabolism, we demonstrated that a major mechanism is induction of PPAR-α. PPAR-α is a key transcription factor promoting lipolysis and lipid oxidation in different tissues[21]. Mice lacking PPAR-α develop obesity and liver steatosis[22]; similarly, the hepatic levels of PPAR-α are decreased in patients with NAFLD[23], and pharmacological agonists are able to improve liver steatosis[24]. Previous studies have suggested that PPAR-α induction is involved in the antisteatotic effect of extracts containing ACNs in different models of obesity[19,20]. Along with the promotion of lipid oxidation, *Moro* juice consumption induced the inhibition of lipogenesis. In this respect, a major mechanism for the antisteatotic effect is the reversion of LXR-α expression. LXR-α is a nuclear hormone receptor that promotes lipogenesis[25], which is increased in the liver of patients with NAFLD[26]. LXR-α stimulates lipogenesis through upregulation of enzymes of *de novo* lipogenesis such as FAS[25]. Suppression of LXR-α expression and activity exerts potent antisteatotic effects in the liver[27]; some flavonoids have been shown to inhibit LXR-α[28].

Besides the effects on lipid homeostasis, the beneficial impact of *Moro* on insulin sensitivity is also noteworthy. Insulin resistance is the metabolic hallmark of patients with NAFLD[29]. Studies using the hyperinsulinemic-euglycemic clamp coupled with infusion tracers have demonstrated that hepatic fat content is directly related to insulin resistance in the liver, skeletal muscle, and adipose tissue of obese subjects[5]. In contrast, recent findings have demonstrated that liver triglyceride content, not visceral adipose tissue volume, predicts the impairment of insulin sensitivity and of very low density lipoprotein secretion in patients with obesity[30,31].

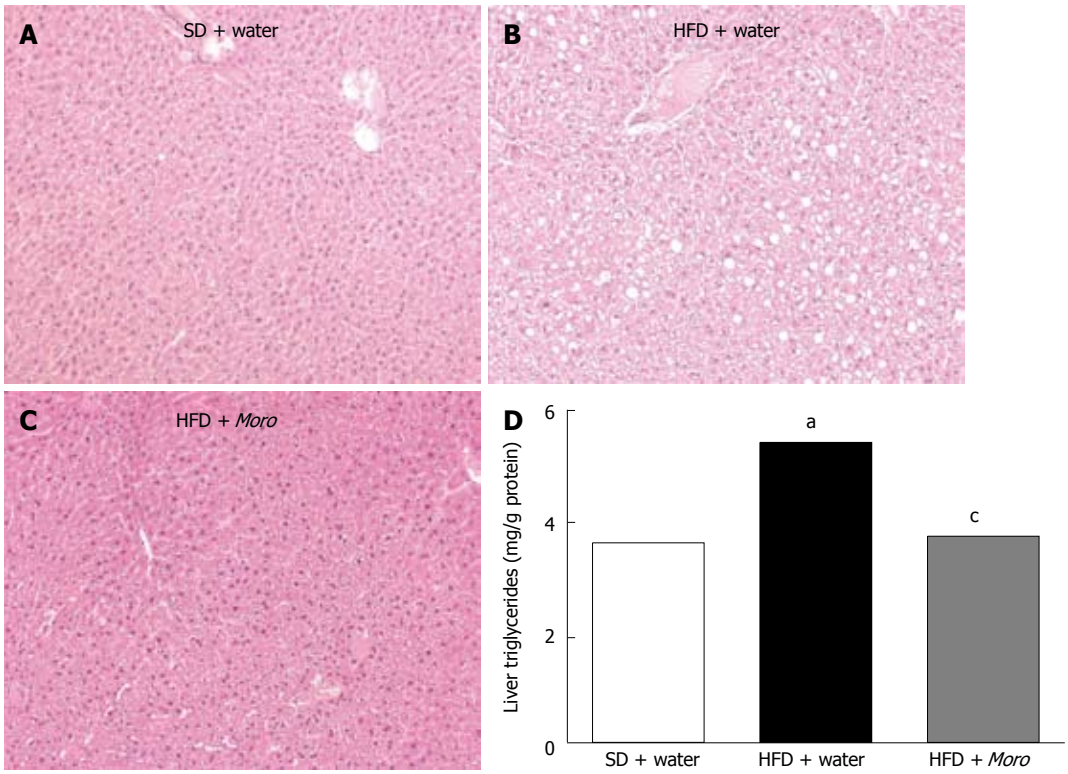

Figure 3 Effects of *Moro* juice on liver histology and liver triglycerides content. A: Hematoxylin-eosin-stained liver sections showing normal histology in lean mice; B: Moderate panacinar steatosis and hepatocyte ballooning in mice fed high-fat diet (HFD); C: Liver sections of mice fed HFD + *Moro* showing absence of steatosis and ballooning; D: Liver triglycerides content was significantly decreased in mice drinking orange juice. $^{a}P < 0.05$ *vs* standard diet (SD) + water, $^{c}P < 0.05$ *vs* HFD + water. Magnification: 10 ×.

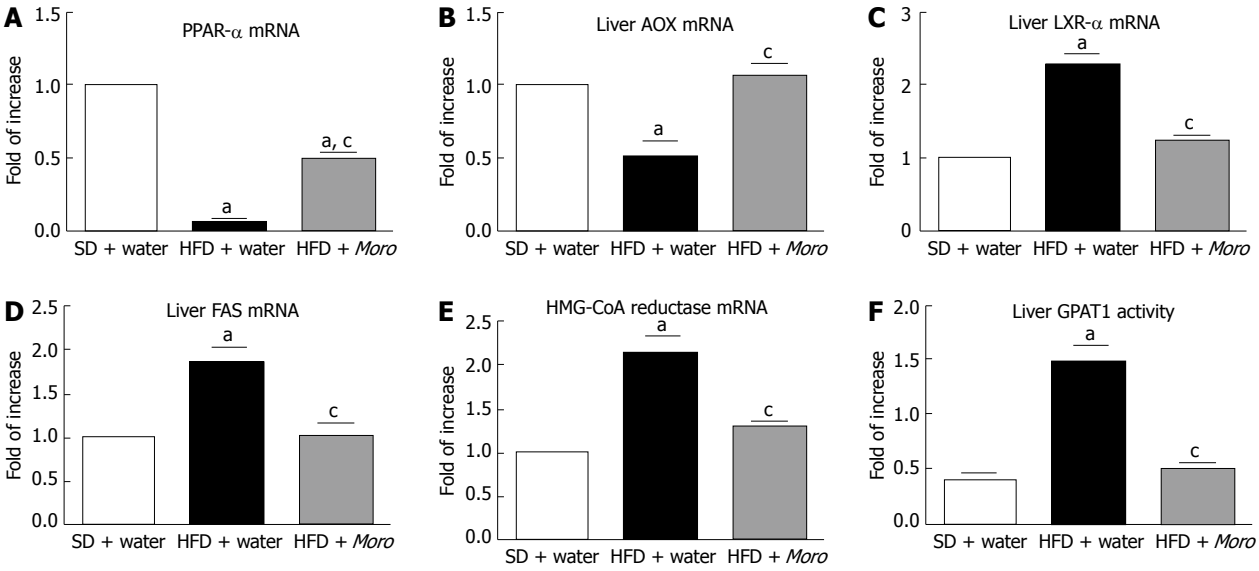

Figure 4 Effects of *Moro* juice on liver lipid homeostasis. Gene expression of (A) peroxisome proliferator-activated receptor (PPAR)-α and (B) acyl-CoA oxidase (AOX) was significantly increased in mice fed high-fat diet (HFD) + *Moro*, whereas gene expression of (C) liver X receptor (LXR)-α, (D) fatty acid synthase (FAS) and (E) hydroxy methylglutaryl (HMG)-CoA reductase was markedly reduced by orange juice; Liver glycerol-3-phosphate acyltransferase 1 (GPAT1) was increased in mice fed HFD + water and was restored to control levels in mice drinking *Moro* juice (F). $^{a}P < 0.05$ *vs* standard diet (SD) + water, $^{c}P < 0.05$ *vs* HFD + water.

Although mice drinking *Moro* juice remained hyperglycemic, probably because of the sugar content, the ITT unequivocally revealed that *Moro* juice exerted insulin-sensitizing activity. An insulin-sensitizing effect has been demonstrated for ACN-rich extracts from bilberry in genetically obese mice[32], and for dietary blueberry in mice fed HFD[33].

In conclusion, in this study, we demonstrated that the

juice of *Moro* exerts metabolic hepatoprotective effects due to changes in the expression of several enzymes involved in lipid homeostasis. Thus, the dietary administration of this food may be effective in preventing liver steatosis, and may be considered as a nutritional approach for the prevention of NAFLD. Clinical trials are now warranted.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder with significant impact on cardiovascular and liver-related mortality. Lifestyle, that is, dietary habits and physical activity, plays a pivot role in the pathogenesis of NAFLD.

Research frontiers

Anthocyanins (ACNs) are water-soluble plant polyphenolic pigments that confer a typical blue, red or purple color, and are contained especially in berries, blood oranges, and pigmented corn and potato. ACNs have been attributed several putative therapeutic roles, including beneficial effects on obesity and related metabolic complications. Diet enriched with ACNs may provide a useful tool to counteract liver steatogenesis.

Innovations and breakthroughs

ACNs are contained in blood oranges, a variety of sweet orange (*Citrus sinensis*) with an intense red pigmentation. A recent study has demonstrated that blood orange consumption inhibits fat accumulation in mice. Furthermore, the administration of protocatechuic acid, the major *in vivo* metabolite of ACN, reduces the activity of lipogenic enzymes in the liver, thus leading to decreased hepatic lipid accumulation. The data in this article demonstrated for the first time that *Moro* juice counteracted liver steatogenesis in mice with diet-induced obesity, through modulation of enzymes involved in lipogenesis and lipid oxidation. Thus, *Moro* juice consumption may represent a promising dietary option for prevention of fatty liver.

Terminology

ACNs are water-soluble pigments that belong to the family of flavonoids. They are contained in berries, blood oranges, and pigmented corn and potato. They are able to modulate lipid and glucose metabolic pathways in humans.

Peer review

It is interesting to readers and may be effective dietary supplements for NAFLD. The manuscript described an interesting finding about the protective effect of this orange juice on the development of fatty liver induced by a high-fat diet in mice.

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BRIEF ARTICLE

A totally mini-invasive approach for colorectal laparoscopic surgery

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Abstract

AIM: To study the short-term outcome of patients treated with laparoscopic right colectomy and how intracorporeal anastomosis has improved the outcome.

METHODS: We retrospectively examined all patients affected by colorectal cancer who underwent a laparoscopic right colectomy between January 2006 and December 2010 in our department. Our evaluation criteria were: diagnosis of colorectal carcinoma at presurgical biopsy, elective surgery, and the same surgeon. We excluded: emergency surgery, conversions from laparotomic colectomy, and other surgeons. The endpoints we examined were: surgical time, number of lymph nodes removed, length of stay (removal of nasogastric tube, bowel movements, gas evacuation, solid and liquid feeding, hospitalization), and major complications. Seventy-two patients were divided into two groups: intracorporeal anastomosis (39 patients) and extracorporeal anastomosis (33 patients).

RESULTS: Significant differences were observed between intracorporeal *vs* extracorporeal anastomosis, respectively, for surgical times (186.8 min *vs* 184.1 min, $P < 0.001$), time to resumption of gas evacuation (3 d *vs* 3.5 d, $P < 0.001$), days until resumption of bowel movements (3.8 d *vs* 4.9 d, $P < 0.001$), days until resumption of liquid diet (3.5 d *vs* 4.5 d, $P < 0.001$), days until resuming a solid diet (4.6 d *vs* 5.7 d, $P < 0.001$), and total hospitalization duration (7.4 d *vs* 8.5 d, $P < 0.001$). In the intracorporeal group, on average, 19 positive lymph nodes were removed; in the extracorporeal group, on average, 14 were removed $P < 0.001$). Thus, intracorporeal anastomosis for right laparoscopic colectomy improved patient outcome by providing faster recovery of nutrition, faster recovery of intestinal function, and shorter hospitalization than extracorporeal anastomosis.

CONCLUSION: Short-term outcomes favor intracorporeal anastomosis, confirming that a less traumatic surgical approach improves patient outcome.

Key words: Anastomosis; Cancer; Colorectal disease; Surgery; Laparoscopy

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INTRODUCTION

Over the last decades, colorectal laparoscopic surgery has outachieved traditional surgery for safety and oncological radicality. Some studies such as COST[1], CLASICC[2], Barcelona[3], and COLOR[4] have shown short-term postoperative advantages without compromising long-term oncological results. For right colectomy, laparoscopic surgery was established later because of technical difficulties related to anatomic and vascular variability[5]. The re-establishment of intestinal transit with ileocolic anastomosis can be performed with two techniques: extracorporeal anastomosis and intracorporeal anastomosis. In extracorporeal anastomosis, also called "laparoscopic-assisted colectomy", stitching (manual or mechanical) and vascular resection are done extracorporeally by externalizing the bowel through a cutaneous mini-incision. On the other hand, the other type of anastomosis is totally intracorporeal, and the stitching is often performed mechanically. A case-control study showed that laparoscopic right colectomy performed with intracorporeal anastomosis is considered one of the most difficult surgeries after transverse resection, rectum resection, and recanalization after Hartmann's resection. However, the ligation of ileocolic vessels and the medial-to-lateral dissection of the right mesocolon intracorporeally are more oncologically safe in terms of the number of lymph nodes removed and cancer management. Nevertheless, extracorporeal anastomosis is the most popular as the literature shows, although it has some limitations in terms of the number of accesses and complications after bowel externalization[6-11].

We evaluated the short-term outcome of patients with colorectal cancer who underwent a laparoscopic right colectomy, and we showed that intracorporeal anastomosis improved patient outcome (shorter hospitalization, fewer postoperative complications, and better oncological radicality). Finally, we considered the influence of the "learning curve" on these surgical techniques.

MATERIALS AND METHODS

We retrospectively examined all patients with colorectal cancer who underwent a laparoscopic right colectomy between January 2006 and December 2010 in our department. Our evaluation criteria were elective surgery and the same surgeon as the first operator. We excluded patients undergoing emergency surgery, conversions from laparotomic colectomy, and other surgeons as the first operator. Patients were divided into two groups: the first included those who underwent a right colectomy with intracorporeal anastomosis, and the second included those who underwent a right colectomy with extracorporeal anastomosis. Patient data included age, gender, body mass index (BMI), American Society of Anesthesiology (ASA) class, and surgical history. We also obtained other data concerning the operation including the surgical time, preoperative diagnosis, and number of lymph nodes removed. We noted parameters of post-surgery hospitalization including removal of nasogastric tube, resumption of bowel movements, resumption of gas evacuation, time to consumption of solid and liquid feeding. We also considered major complications in terms of post-surgery time and hospitalization.

Table 1 Homogeneous groups

Patients		Intracorporeal $n = 39$	Extracorporeal $n = 33$	
Age (yr)	Median	74.5	74	NS
	Min-max	53-89	45-96	
Gender	Male	24	20	NS
	Female	15	13	
	Ratio M/F	1.6	1.5	
Weight (kg)	Median	71	77	NS
	Min-max	50-90	51-120	
Height (cm)	Median	165	167	NS
	Min-max	148-182	146-183	
BMI (kg/m^2)	Median	26.3	28.1	NS
	Min-max	20-37	19.9-37	

BMI: Body mass index; M: Male; F: Female; NS: Not significant.

We obtained data from medical records, surgical cards, and databases. We used JMP software 7a Version [SAS Institute Inc. (1989-2007), Cary, NC, United States] for electronic data processing. Descriptive variables were expressed as mean, standard deviation, mode, median, number of events, patients, and percentage. According to the different features of these variables, we used the χ^2 test, F test, and Student's t test as appropriate, and considered $P < 0.05$ to indicate statistical significance.

RESULTS

In this study, 72 patients were divided into two groups: intracorporeal anastomosis ($n = 39$) and extracorporeal anastomosis ($n = 33$). There were no significant differences in age, gender, BMI (Table 1), or ASA class between the two groups ($P = 0.8645$ for ASA).

Twenty patients in the intracorporeal group had a positive abdominal surgical history (51.3%), whereas 21 patients in the extracorporeal group had such a history (63.6%; $P = 0.8433$, Table 2). There were also no significant differences in the diagnosis from the pre-surgery biopsy (Figure 1).

In the intracorporeal group, we performed additional surgical procedures in seven patients during surgery, i.e., one nefrectomy, five colecistectomies, and one intraoperative coloscopy, whereas in the extracorporeal group, we performed two colecistectomies, one intraoperative coloscopy, and one polipectomy.

In the intracorporeal group, we removed an average of 19 lymph nodes (range: 7-36), whereas in the extracorporeal group we removed an average of 14 lymph nodes (range: 2-29, $P < 0.0001$).

The average surgical time was 186.8 min (range: 105-280 min) in the intracorporeal group and 184.1 min in the extracorporeal group (range: 115-285 min, $P = 0.6549$).

Gas evacuation was shorter in the intracorporeal

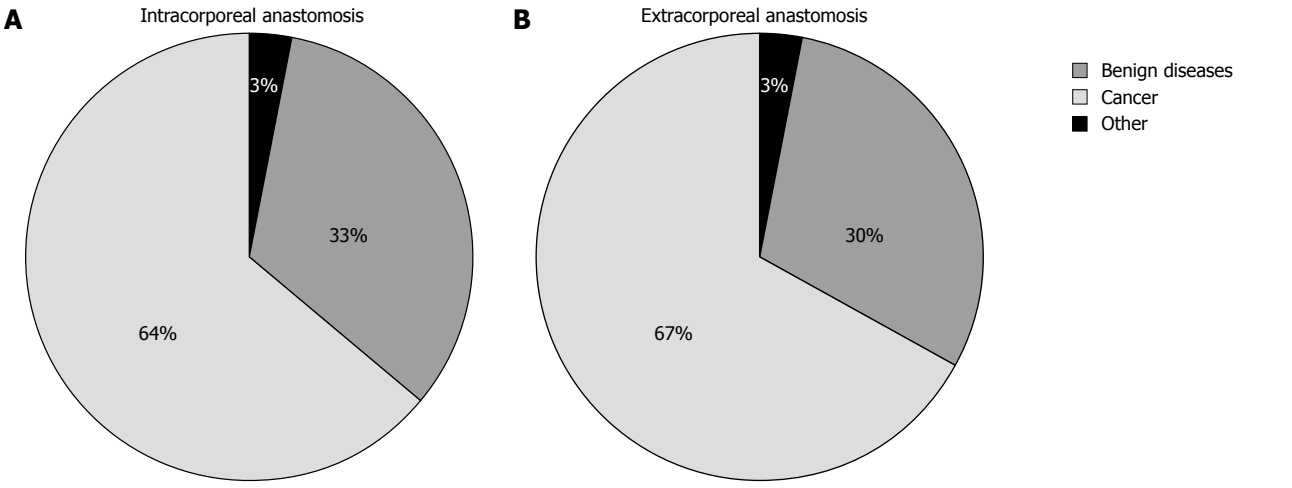

Figure 1 Intracorporeal anastomosis and extracorporeal anastomosis were no significant differences in the diagnosis from the pre-surgery biopsy. A: Intracorporeal anastomosis; B: Extracorporeal anastomosis.

Table 2 Abdominal surgery history

Intracorporeal anastomosis		Extracorporeal anastomosis	
20/39 (51.3%)		21/33 (63.6%)	
One operation	11	One operation	14
Two operations	7	Two operations	7
> Two operations	2	> Two operations	7
Appendectomy	9	Appendectomy	15
Colic resection	2	Colic resection	1
Isteroannessectomy	4	Isteroannessectomy	2
Cholecystectomy	5	Cholecystectomy	1
Urologic surgery	4	Urologic surgery	1
Hernioplasty	2	Hernioplasty	9

Table 3 Length of stay

		Intracorporeal anastomosis	Extracorporeal anastomosis	*P* value
Time until resumption of gas evacuation (d)	Median	3	3.5	< 0.0001
	Min-max	1-6	1-6	
Time until resumption of bowel movements (d)	Median	3.8	4.9	< 0.0001
	Min-max	1-7	2-7	
Time until removal of nasogastric tube (d)	Median	1.8	3	< 0.0001
	Min-max	0-11	0-6	
Time until resumption of liquid diet (d)	Median	3.5	4.5	< 0.0001
	Min-max	2-12	2-10	
Time to resumption of solid diet (d)	Median	4.6	5.7	< 0.0001
	Min-max	2-12	3-11	
Discharge (d)	Median	7.4	8.5	NS
	Min-max	4-19	5-25	0.5424

NS: Not significant.

group than in the extracorporeal group (3 ± 1.05 d *vs* 3.5 ± 1.1 d, range: 1-6 d, $P < 0.0001$). Bowel movements occurred earlier in the intracorporeal group (3.8 ± 1.4 d, range: 1-7 d *vs* 4.9 ± 1.5 d, range: 2-8 d, $P < 0.0001$). In addition, the NGT was removed sooner in the intracorporeal group than in the extracorporeal group (1.8 d and 3 d, respectively, $P < 0.0001$).

Resumption of a liquid diet occurred an average of (3.5 ± 2.2 d, range: 2-12 d, $P < 0.0001$) after intracorporeal anastomosis and (4.5 ± 1.7 d, range: 2-10 d, $P < 0.0001$) after extracorporeal anastomosis. Resumption of a solid diet occurred (4.6 ± 2.1 d, range 2-12 d) and after intracorporeal and extracorporeal anastomosis, respectively (5.7 ± 1.7 d, range: 3-11 d, $P < 0.0001$).

Total hospitalization time was significantly less after intracorporeal anastomosis (average of 7.4 ± 3.2 d, range: 4-19 d) than after extracorporeal anastomosis (average of 8.5 ± 3.9 d, range: 5-25 d, $P < 0.0001$; Table 3).

Major complications occurred in 10.2% of patients undergoing intracorporeal anastomosis, i.e., three patients: one with severe anemia, one with anastomotic dehiscence, and one with enterocutaneous fistula. In the extracorporeal group, 12.1% of patients had major complications, i.e., five patients: two with severe anemia, one with occlusion, one with anastomotic dehiscence, and one with enterocutaneous fistula.

DISCUSSION

Our study shows that intracorporeal anastomosis for right laparoscopic colectomy improved patient outcome compared with patients who underwent extracorporeal anastomosis. With intracorporeal anastomosis, we found faster recovery of nutrition, faster recovery of intestinal function, and shorter hospitalization. However, there was no difference in average surgery time between the two groups.

According to the inclusion and exclusion criteria, we obtained two homogeneous and comparable groups without significant differences in age, gender, BMI (Tables 1 and 4), ASA class, or abdominal surgical history (Table 2). In laparoscopic right colectomy with extracorporeal anastomosis (laparoscopic-assisted colectomy), the bowel is externalized through a lateral mini-incision. With this approach, bowel mobilization and ligation of vessels is usually laparoscopic, whereas resection of the specimen and creation of the anastomosis is extracorporeal. On the other hand, in laparoscopic right colectomy with intracorporeal anastomosis (totally laparoscopic

Table 4 Patient distribution according to age and body mass index, number of removed lymph nodes and duration of hospital stay *n* (%)

	Intracorporeal	Extracorporeal
Age (yr)		
< 65	13.6	12.1
65-80	43.2	48.5
> 80	43.2	33.4
BMI (kg/m^2)		
< 25	44.4	26.9
25-30	40.7	53.1
> 30	14.9	23.0
Number of lymph nodes removed		
< 12	7 (21.2)	14 (46.7)
12-15	6 (18.2)	6.6 (2)
> 15	20 (60.6)	14 (46.7)
Discharge from hospital (d)		
< 6	15 (42.8)	8 (24.4)
7	10 (28.6)	10 (30.3)
8-9	5 (14.3)	8 (24.2)
> 10	5 (14.3)	7 (21.1)

BMI: Body mass index.

colectomy), bowel mobilization, ligation of vessels, resection of the specimen, and creation of the anastomosis are totally intracorporeal.

In our experience, right colectomy with intracorporeal anastomosis has been standardized step by step: first, ileocolic vessels are isolated, secured between clips, and divided near their origin. Then, the right mesocolon is dissected medial-to-lateral, and the small bowel mesentery is divided to reach the edge of the terminal ileum. Then, the specimen is resected with an Endo-GIA stapler. The end the Endo-GIA stapler is deployed through the bowel openings to form a side-to-side anastomosis. The last step is specimen extraction and wound closure. We did not standardize the right colectomy with extracorporeal anastomosis; in 36.4% of cases, the ligation of vessels was performed after partial bowel mobilization, whereas in 63.6% of cases, it was the first step of the surgical procedure. Finally, the anastomosis was realized manually, lateral-to-lateral, in a double layer.

Both techniques are oncologically safe; according to the latest Union for International Cancer Control Tumor Node Metastasis classification, removal of at least 12 lymph nodes is fundamental to guarantee sufficient oncological radicality[12].To achieve this goal, the arterial vessels must be ligated at the origin from the superior mesenteric artery. When vascular ligation is extracorporeal, it is very difficult to obtain an adequate number of lymph nodes[13]. Bergamaschi *et al*[14] showed that extracorporeal vascular oncologic ligation is very difficult through a small cutaneous incision, and the bowel undergoes a hard traction with this technique. Hellan *et al*[15] emphasized that the limitations of extracorporeal vascular ligation include poor exposure of the ileocolic pedicle through the small incision. Difficult exposure of the base of the mesentery could compromise the oncological result. That is why some surgeons propose the technique of intracorporeal high-vessel ligation combined with extracorporeal anastomosis[16-19].

Regarding oncological radicality, we found significant differences in the number of lymph nodes removed. We removed an average of 19 lymph nodes from the intracorporeal group and 14 lymph nodes from the extracorporeal group. In particular, in the first group we removed more than 15 lymph nodes in 60% of patients, 12 to 15 lymph nodes in 18.2% of patients, and fewer than 12 lymph nodes in 21% of patients. In the extracorporeal group, we removed more than 15 lymph nodes in 46.7% of patients, 12 to 15 lymph nodes in 6.6% of patients, and fewer than 12 lymph nodes in 46.7% of patients (Table 4). Thus, our experience shows that there is an important difference in the number of positive lymph nodes removed in the intracorporeal group, and also on the percentage of patients in which more than 12 lymph nodes were removed ($P < 0.0001$). The explanation for this difference is the missed ligation of vessels before the mobilization of the right colon. We believe that is very difficult to obtain an adequate number of lymph nodes when vessel division is not the first step in laparoscopic right colectomy.

In the literature, some authors have reported no differences in safety, whereas others noted that the only advantage was a smaller incision[20,21]. On the other hand, other studies affirmed the safety of intracorporeal anastomosis, with the same complication rate as for extracorporeal anastomosis[22,23].

Because intracorporeal anastomosis is considered more difficult, only a few surgeons have used this kind of technique; however less mobilization is required, and less tension is applied to the bowel and mesentery because the bowel does not need to reach the anterior abdominal wall for externalization. Furthermore, the excessive tension on the mesentery during the mobilization is associated with an increased risk of mesenteric or portal vein thrombosis[24].

Concerning surgical times, we did not find a significant difference in surgical time between the two groups.

Patients in the intracorporeal group had a shorter hospitalization duration. In some cases, the hospitalization duration was longer possibly because of age (43.2% of patients in the intracorporeal group and 33.4% in the extracorporeal group were over 80 years old). Our results showed a significantly shorter average hospitalization stay in the intracorporeal group (Table 4). These data agree with a recent Spanish study[25], although this difference was not significant ($P = 0.5424$) because hospitalization duration is influenced by many patient factors. On the other hand, we found that 71.4% of patients in the intracorporeal group went home within 7 d, and 54.7% of patients in the extracorporeal group went home within this period ($P = 0.0001$, Figure 2).

Concerning the recovery of intestinal function, our results found significantly shorter average times for resumption of gas evacuation after 3 d in the intracorporeal group compared to after 3.8 d in the extracorporeal

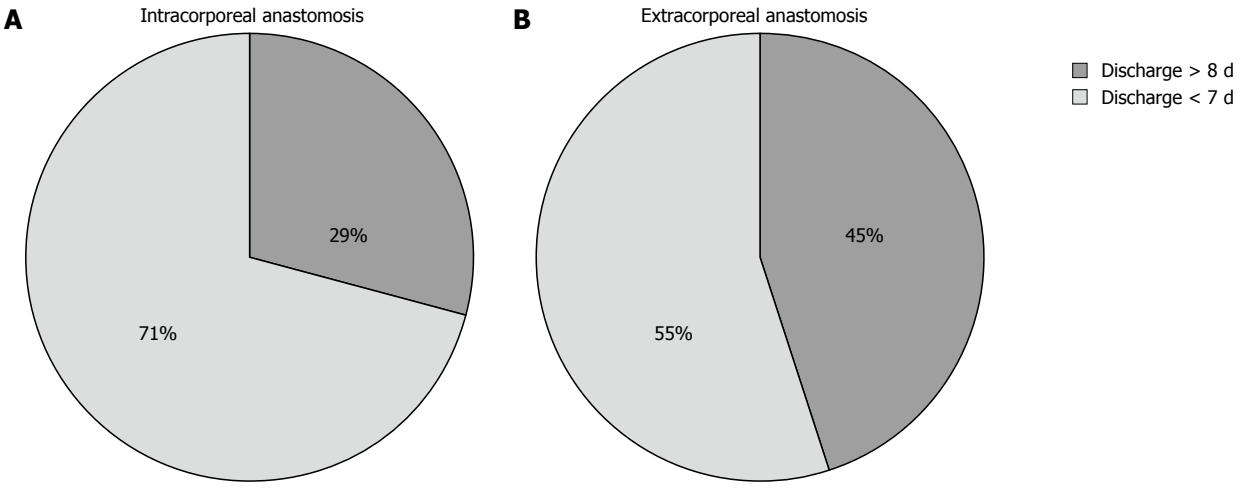

Figure 2 Patients in the intracorporeal group and extracorporeal group went home within 7 d. A: Intracorporeal group; B: Extracorporeal group.

group. Bowel movements occurred after an average of 4.9 d in the intracorporeal group. In the intracorporeal group, the nasogastric tube was removed after 1.8 d, whereas it was removed after 3 d in the extracorporeal group. This difference can be explained by an increased percentage of paralytic ileum in the second group, which is due to the traction of the right colon and terminal ileum through the mini-incision on the pancreas and duodenum[26]. This approach allowed a more rapid recovery of liquid and solid nutrition consumption.

We analyzed major complications, which included severe anemia, occlusion, anastomotic dehisces, and enterocutaneous fistulae. There were no significant differences between the two groups.

In conclusion, our study clearly shows that laparoscopic right colectomy with intracorporeal anastomosis improves patient outcome. We found that intracorporeal anastomosis resulted in faster recovery of nutrition consumption, faster recovery of intestinal function, and shorter hospitalization duration. The higher number of lymph nodes removed seems to be related to vascular division as the first surgical step as a rule. This confirms that a mini invasive approach improves patient outcome.

COMMENTS

Background

A lot of studies have demonstrated the benefits of laparoscopic right colectomy, proving the short-term outcome of patients. The aim of this study is to show how intracorporeal anastomosis in laparoscopic right colectomy has further improved patient outcome.

Research frontiers

In the area of mini-invasive surgery, intracorporeal anastomosis, confirm that a less traumatic surgical approach improves patient outcome.

Innovations and breakthroughs

Based on a large series, this study describes the outcome of patients treated with laparoscopic right colectomy and is a reference for comparison in future studies.

Applications

The study results show how laparoscopic right colectomy with intracorporeal anastomosis improves patients outcome. This study suggest that all patients treated with intracorporeal anastomosis have faster recovery of nutrition consumption, faster recovery of intestinal function, and shorter hospitalization duration.

Peer review

This paper demonstrated the outcomes of patients treated with laparoscopic right colectomy with intracorporeal anastomosis.

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BRIEF ARTICLE

A novel animal model for *in vivo* study of liver cancer metastasis

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Abstract

AIM: To establish an animal model with human hepatocyte-repopulated liver for the study of liver cancer metastasis.

METHODS: Cell transplantation into mouse livers was conducted using alpha-fetoprotein (AFP)-producing human gastric cancer cells (h-GCCs) and h-hepatocytes as donor cells in a transgenic mouse line expressing urokinase-type plasminogen activator (uPA) driven by the albumin enhancer/promoter crossed with a severe combined immunodeficient (SCID) mouse line (uPA/SCID mice). Host mice were divided into two groups (A and B). Group A mice were transplanted with h-GCCs alone, and group B mice were transplanted with h-GCCs and h-hepatocytes together. The replacement index (RI), which is the ratio of transplanted h-GCCs and h-hepatocytes that occupy the examined area of a histological section, was estimated by measuring h-AFP and h-albumin concentrations in sera, respectively, as well as by immunohistochemical analyses of h-AFP and human cytokeratin 18 in histological sections.

RESULTS: The h-GCCs successfully engrafted, repopulated, and colonized the livers of mice in group A (RI = 22.0% ± 2.6%). These mice had moderately differentiated adenocarcinomatous lesions with disrupted glandular structures, which is a characteristics feature of gastric cancers. The serum h-AFP level reached 211.0 ± 142.2 g/mL (range, 7.1-324.2 g/mL). In group B mice, the h-GCCs and h-hepatocytes independently engrafted, repopulated the host liver, and developed colonies (RI = 12.0% ± 6.8% and 66.0% ± 12.3%, respectively). h-GCC colonies also showed typical adenocarcinomatous glandular structures around the h-hepatocyte-colonies. These mice survived for the full 56 day-study and did not exhibit any metastasis of h-GCCs in the extrahepatic regions during the observational period. The mice with an h-hepatocyte-repopulated liver possessed metastasized h-GCCs and therefore could be a useful humanized liver animal model for studying liver cancer metastasis *in vivo*.

CONCLUSION: A novel animal model of human liver cancer metastasis was established using the uPA/SCID mouse line. This model could be useful for *in vivo* testing of anti-cancer drugs and for studying the mechanisms of human liver cancer metastasis.

Key words: Urokinase-type plasminogen activator/severe combined immunodeficient mouse; Mouse with humanized liver; Liver cancer metastasis; Alpha-fetoprotein-producing gastric cancer cells

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INTRODUCTION

Tumor metastasis, which is defined by a process in which tumor cells originating from an organ invade another anatomically distant organ, is the leading cause of cancer-related mortality[1,2]. One of the major target organs for cancer metastasis is the liver[1-3], and therefore there is increasing need for animal models that accurately mimic the pathophysiological situations in human liver and are suitable for investigating the mechanisms of hepatic cancer metastasis. In fact, several studies have attempted to transplant metastatic h-tumor cells into the livers of the immuno-compromized mice, such as athymic nude mice[4], which cannot generate T cells, severe combined immunodeficient (SCID) mice that lack mature B and T cells[5-7], and NOD/SCID/$_c^{null}$ (NOG) mice[8,9], which are deficient in T, B, and natural killer cells, and have impaired dendritic cells. In these animal models, the transplanted h-tumor cells invade the hepatic parenchyma, which is composed of mouse hepatocytes that are phylogenetically distant from h-hepatocytes and are known to exhibit biological and pathological features that are different from the human counterpart.

Heckel *et al*[10] established transgenic mice expressing urokinase type plasminogen activator (uPA) under the control of the albumin (Alb) enhancer/promoter and found that the m-hepatocytes were constitutively damaged due to constant exposure to the expressed uPA. In another study, a mouse line possessing a humanized liver (chimeric mouse) was generated by transplanting healthy and normal h-hepatocytes into the liver of the immuno- and liver-compromized mouse, which was created by mating the uPA-Tg mouse with the SCID mouse (uPA/SCID mouse)[10,11].

We previously developed chimeric mice where the liver was stably and reproducibly replaced with h-hepatocytes and found that the occupancy ratio or replacement index (RI) in the parenchyma was quite high (> 90%) in best cases[12]. Human hepatocytes in the chimeric m-liver have been intensively and extensively characterized based on normal hepatic phenotypes, such as expression profiles of cytochrome P450, the major xenobiotic-metabolizing enzymes, drug-metabolizing capacities, and hepatitis virus infectivity[11,13-15]. Based on these studies, which indicate that a chimeric m-liver can appropriately recapitulate the characteristics of h-liver, we hypothesized that the chimeric mouse as an animal model can be used to investigate the underlying mechanisms of tumor metastasis into the liver where the parenchyma is largely composed of normal and healthy h-hepatocytes.

In the present study, we established a chimeric mouse as a novel experimental model that sufficiently mimics the pathophysiological micro-environment in h-liver for studying liver cancer metastasis.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the National Hospital Organization, Nagasaki Medical Center, the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board, and the Phoenix-Bio Ethics Board. This study was conducted in accordance with their guidelines.

Animals

The uPA/SCID mice were generated and used as transplant hosts once they reached an age of 24-32 d old as previously described[14,15]. The mice were maintained in the laboratory in a specific pathogen-free environment in accordance with the guidelines of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board as well as the PhoenixBio Ethics Board.

Cancer cells

Human gastric cancer cells (h-GCCs) were purchased from the Japanese Collection of Research Biosources (Osaka, Japan) and used as liver metastatic cancer cells. These cells are adenocarcinoma cells derived from human gastric cancer cells that produce alpha-fetoprotein (AFP) and have a high affinity for liver tissue[16-18]. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, United States) containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, United States) in an atmosphere of 95% air and 5% CO_2 at 37 ℃.

Cell transplantation into the uPA/SCID

Human GCCs were suspended at a concentration of 1 × 10^7 cells/mL and placed on ice until transplantation. Cryopreserved h-hepatocytes derived from a 6-year-old African female were purchased from BD Biosciences (San Jose, CA, United States), thawed in a 37 ℃ water bath, rapidly diluted with culture medium at 4 ℃, and washed twice to remove the cryopreservation solution. The cell viability was assessed by a trypan blue exclusion test. The uPA/SCID mice were anesthetized with ether and then were intrasplenically injected with the h-hepatocytes as previously described[12]. Blood samples, 5 μL each, were periodically collected from the host tail-vein for

Table 1 Serum concentrations of human albumin and human alpha-fetoprotein in host mice at 56 d post-transplantation

Experimental groups	Transplanted cells	No. of animals	Serum concentration	
			h-Alb (mg/mL)	h-AFP (mg/mL)
A	h-GCCs	4	UD	7.1-324.2 (211.0 ± 142.2)
B	h-GCCs and h-hepatocytes	6	0.03-9.1 (3.1 ± 3.5)	0.3-126.1 (54.3 ± 60.7)

The numerals represent the range of the concentrations and those in the parentheses indicate the mean ± SD. h-GCCs: Human gastric cancer cells; h-Alb: Human albumin; h-AFP: Human alpha-fetoprotein; h-hepatocytes: Human hepatocytes; UD: Undetectable.

determining concentrations of human albumin (h-Alb) and human AFP (h-AFP) using an h-Alb enzyme-linked immunosorbent assay quantification kit (Bethyl Laboratories Inc., Montgomery, TX) and an h-AFP enzyme immunoassay test kit (Hope Laboratories, Belmont, CA, United States), respectively.

Histological and immunohistochemical evaluation of the m-liver

Liver tissue specimens were removed from the transplanted mice, paraffin-embedded, sectioned at a 4 μm thickness, and stained with hematoxylin and eosin (H and E). Human hepatocyte-colonies were identified by staining the sections with mouse monoclonal antibodies against human-specific cytokeratin 18 (h-CK18) (DAKO, Glostrup Denmark). Human GCCs in the m-liver were identified by h-AFP staining with a polyclonal Ab (Novocastra Laboratories Ltd, United Kingdom). The sections were treated with a biotinylated, goat anti-rabbit IgG for h-CK18 and rabbit anti-m-IgG (DAKO, Glostrup Denmark) for h-AFP. All of the tissue specimens or cells were counterstained with H and E.

Determination of h-hepatocytes and h-GCCs repopulation of the uPA/SCID m-liver

Serial liver sections were double immunostained for h-CK18 and h-AFP to identify h-hepatocytes/h-GCCs and h-GCCs, respectively. The extent of repopulation of h-hepatocytes and h-GCCs in the chimeric mouse liver was determined as the RI, which is the occupational ratio of the transplanted cells in the examined area of histological sections, as previously described[12]. The RI of h-hepatocytes ($RI_{h\text{-}hepatocytes}$) in the uPA/SCID m- liver was determined using h-CK18 as a maker to histologically identify h-hepatocytes. When appropriate, the RI for h-GCCs ($RI_{h\text{-}GCCs}$) was referred to as the metastatic index ($MI_{h\text{-}GCCs}$) in this study. Human hepatocytes and h-GCCs were identified on histological sections as the h-CK18-positive (h-CK18$^+$) and h-AFP-negative (h-AFP$^-$) cells and the h-CK18$^+$ and h-AFP$^+$ cells, respectively. The $RI_{h\text{-}hepatocytes}$ and $MI_{h\text{-}GCC}$ of the m- livers were calculated as the ratio of the "h-CK18$^+$/h-AFP$^-$" and "h-CK18$^+$/h-AFP$^+$" areas to the entire examined area of the sections, respectively.

Experimental groups

The uPA/SCID mice were divided into two groups (A and B groups). Four uPA/SCID mice in group A were each injected with 1×10^6 h-GCCs. Six mice in group B were co-transplanted with 7.5×10^5 h-hepatocytes and h-GCCs each. The blood h-Alb and h-AFP concentrations were periodically monitored after cell transplantation. The mice were euthanized at the termination of the experiments and their livers, spleens, and lungs were microscopically examined to identify any metastasis of h-GCCs.

RESULTS

Group A experiment

Human GCCs were transplanted into the livers of uPA/SCID mice and euthanized 56 d after transplantation. Human GCC colonies were macroscopically distinguishable from the host m-liver cells as brown colored regions (Figure 1A). Histological examinations showed that these areas contained h-GCC colonies and host m-liver cells composed of m-parenchymal and m-nonparenchymal cells (Figure 1B). The whitish or pale regions observed in Figure 1A were composed of only m-liver cells. The specimens were also stained for h-AFP to define h-GCCs (Figure 1C and D). Human GCCs formed colonies with well-developed glandular structures, which is a characteristic feature of gastric cancer. The serum concentrations of h-AFP increased to 211.0 ± 142.2 g/mL (range 7.1-324.2 g/mL, Table 1), which reflected the repopulation of h-GCCs in the liver, since serum h-AFP was undetectable in uPA/SCID mice without transplantation of h-GCCs (data; not shown). The MI of h-GCCs ($MI_{h\text{-}GCC}$) was 22.0% ± 2.6% at the termination of the experiment 56 d post-transplantation.

Group B experiment

Both h-hepatocytes and h-GCCs were simultaneously transplanted into six uPA/SCID mice. The serum concentrations of h-Alb and h-AFP monitored after the cell transplantation (Figure 2). These protein levels were variable among individual mice, and three mice (No. 1-3) had substantially elevated h-Alb levels over the 56-d study. In addition, these mice exhibited $RI_{h\text{-}hepatocytes} > 70\%$ based on the correlation graph between h-Alb concentrations and $RIs_{h\text{-}hepatocytes}$[12]. These hosts also had markedly elevated h-AFP concentrations. In particular, mice No. 1 and 2 showed the highest h-Alb levels (approximately 9.1 mg/mL) and h-AFP concentrations (approximately 126.1 mg/mL) at 56 d post-transplantation (Table 1; Figure 2). As shown in Figure 3A, mouse 1 had the highest h-Alb and h-AFP levels, and the liver was composed of brown and whitish regions indicated by the thick and the thin arrows, respectively, which corresponded to the colonies composed of both h-hepatocytes and h-GCCs or m-liver cells, respectively. The brown region in the liver shown in Figure 3A was sectioned and stained with H and E (Figure 3B), anti-h-CK18 Abs to identify both h-hepatocytes and

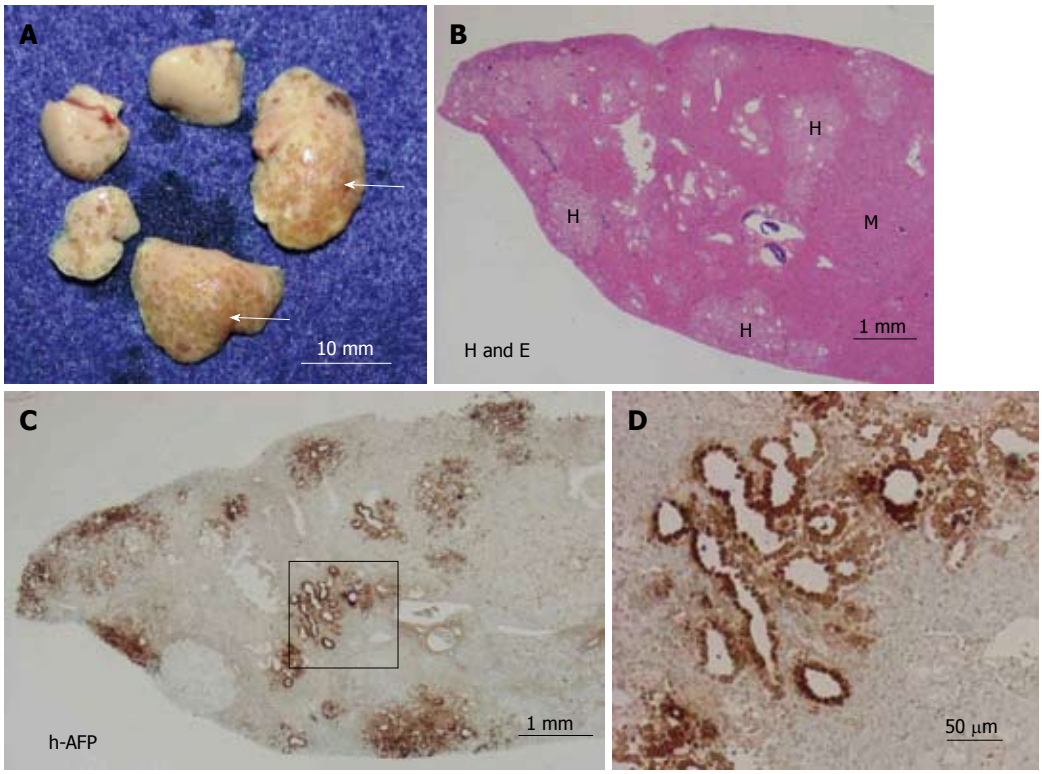

Figure 1 Macro- and microscopic images of the liver from group A mice. A: The urokinase-type plasminogen activator/severe combined immunodeficient mouse mice were transplanted with human gastric cancer cells (h-GCCs) and euthanized 56 d later, at which time the livers were isolated and photographed; B: The arrows in A point to concentrated regions of h-GCC colonies, and the sections were stained with hematoxylin and eosin (H and E). H and M in B represent h-GCC colonies and m-liver cell regions, respectively; C: The sections were stained with anti-human alpha-fetoprotein (h-AFP) antibodies; D: The square region in C is enlarged and shown.

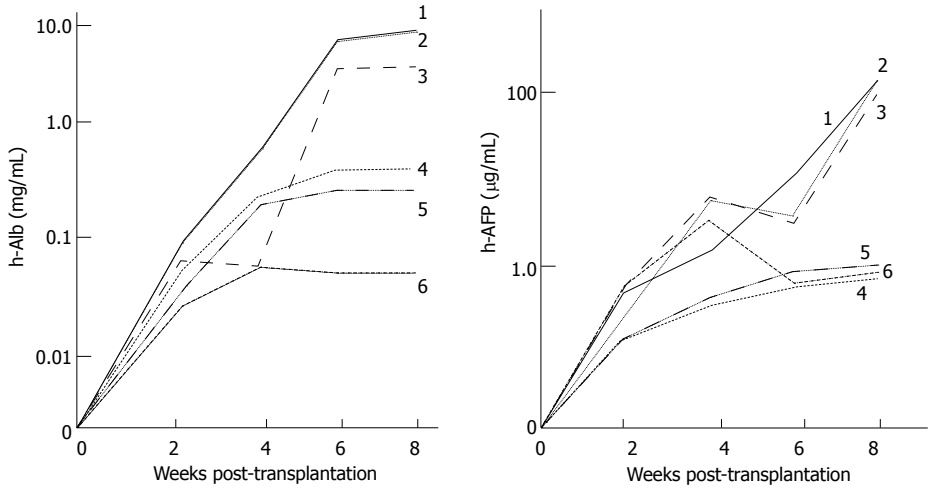

Figure 2 Changes in the serum concentrations of human albumin and human alpha-fetoprotein in group B-mice. Six mice (No.1-6) were co-transplanted with h-hepatocytes and human gastric cancer cells. The serum levels of human albumin (h-Alb) (left panel) and human alpha-fetoprotein (h-AFP) (right panel) were periodically monitored after the cell transplantation.

h-GCCs (Figure 3C), and the anti-h-AFP Ab to identify h-GCCs (Figure 3D). A comparison of Figure 3B and C showed that most of the section from Figure 3B was occupied with h-CK18^{+} cells, which corresponded to the cells in the less eosinophilic areas of the H and E section. Human CK18^{-} m-liver cells were located in eosinophilic areas in the H and E section, which were sporadically distributed as clusters with variable forms among large engrafted h-cell colonies. Human-AFP^{+} h-GCC-colonies were distinguished by comparing Figure 3B-D. These colonies were surrounded with less eosinophilic h-hepatocytes (Figure 3D) that were swollen and clearer (Figure 3B and C). Magnified views of the brown area obtained from another serial sections of the liver shown in Figure 3A are shown in Figure 4A (H and E) and Figure 4B (h-AFP-stain). Human GCCs formed moderately differentiated adenocarcinomas with disrupted glandular structures, which is a characteristic feature of gastric cancer. Morphometric analyses using these h-CK18- and h-AFP-stained serial sections indicated that the $RI_{h\text{-}hepatocyte}$ and $MI_{h\text{-}GCC}$ in group B mice was 66.0% ± 12.3% (n = 6) and 12.0% ± 6.8% (n = 6), respectively. The mice in

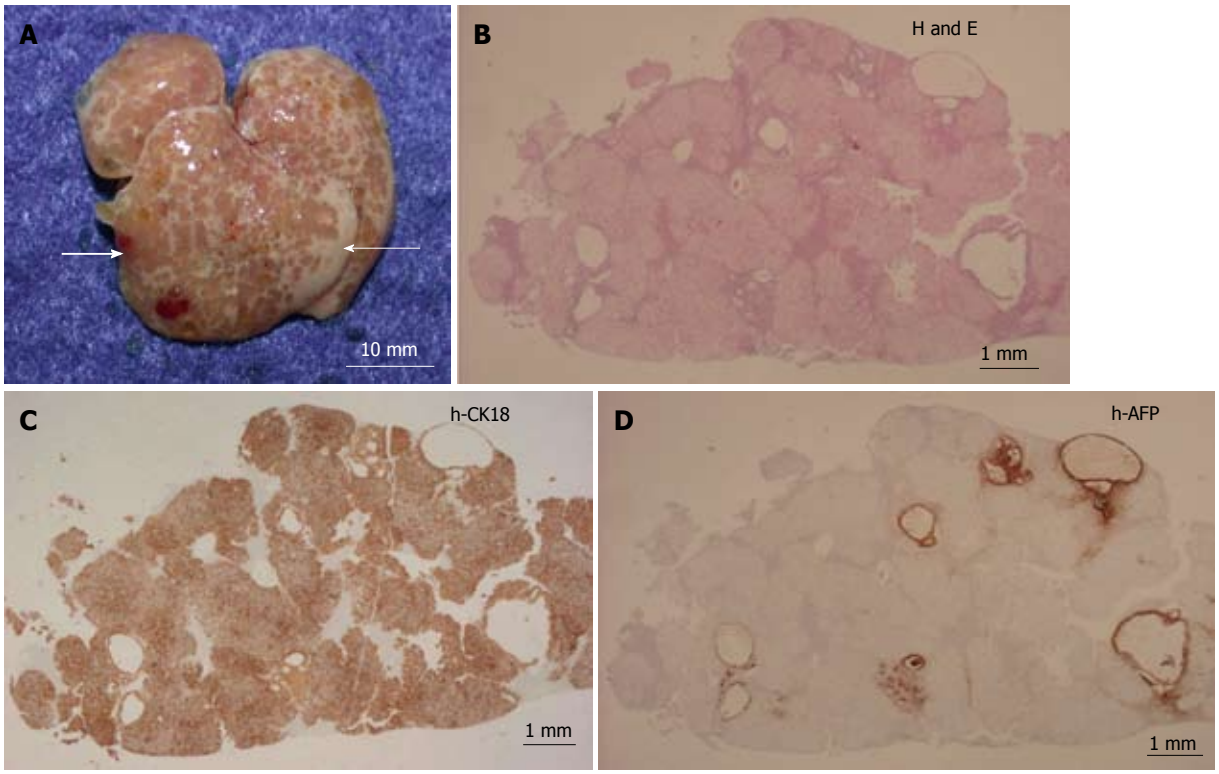

Figure 3 Macroscopic image of the liver of mouse No. 1 from Figure 2 at 56 d post-transplantation. A: The thick and thin white arrows point to h-cells [human hepatocytes (h-hepatocytes) and human gastric cancer cells (h-GCCs)] and m-liver cell regions, respectively; B: The liver was sectioned and stained with hematoxylin and eosin (H and E); C: The liver was sectioned and stained with anti-h-CK18; D: The liver was sectioned and stained with anti-human alpha-fetoprotein (h-AFP) antibodies. The h-AFP + (h-GCC) colonies were surrounded by less eosinophilic h-hepatocytes.

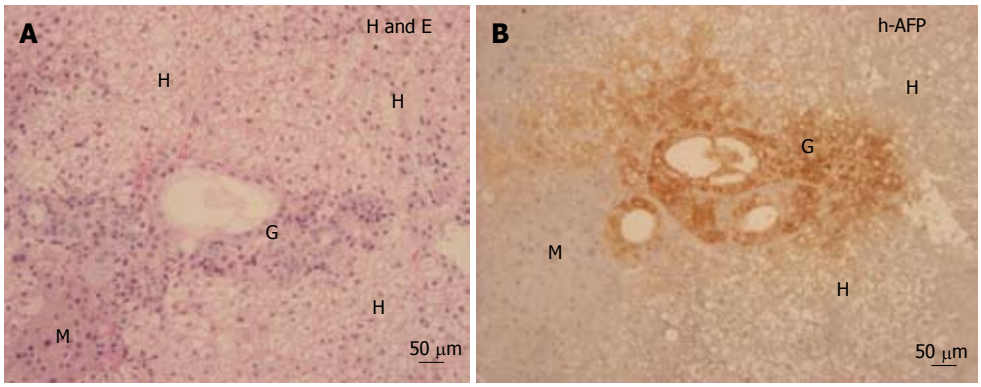

Figure 4 Magnified images of hepatic histology from group B mice. A: A serial section of the liver in Figure 3 was subjected to hematoxylin and eosin (H and E); B: A serial section of the liver in Figure 3 was subjected to human alpha-fetoprotein (h-AFP) staining. H, G and M represent the areas occupied by human-hepatocytes, human gastric cancer cells (h-GCCs), and host m-liver cells, respectively. h-GCCs composed moderately differentiated adenocarcinoma with disrupted glandular structures.

group B survived for the entire 56 d study. Extrahepatic sites and organs, such as the peritoneal cavity and kidney, were also examined for the presence of metastatic h-GCC lesions. The metastatic h-GCCs were not found in the extrahepatic regions during the observational period, indicating that the cells did not metastasize to any other regions.

DISCUSSION

An ideal animal model for liver metastasis of h-cancer cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize in the host liver. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the h-liver *in vivo*. Most of the conventional models to date manifest the first feature, but none of them have been able to sufficiently recapitulate the microenvironment of the h-liver [4-6]. In the present study, we established a unique and novel that possessed both of these features.

In our study, we successfully engrafted the liver with

h-GCCs in the group A mice, and the cells formed relatively large colonies, with the MI as high as 25% at 56 d post-transplantation. However, such a considerably high MI could be a result of effects from either the donor or host side of the model. We chose h-AFP$^+$ h-GCCs as a metastatic cancer cell line, since previous studies reported that patients with AFP$^+$ gastric cancer showed a higher liver MI than those with AFP$^-$ cells; more than 70% of the patients developed liver metastasis[18,19]. These AFP$^+$ cancer cells express c-Met[19], which is the receptor for human hepatocyte growth factor (HGF), and therefore it is plausible that the cells have a high affinity for liver tissues under conditions where the levels of activated HGF in these tissues become high [20]. In the present study, we utilized the uPA/SCID mice as hosts, which possessed a uPA transgene product that continuously damages the hepatocytes. In this model, the host hepatocytes generate pro-inflammatory environments in the liver, which stimulates the mobilization and expression of HGF in the liver tissues, including hepatocytes.

The role of uPA is an important aspect in this model. The host m-hepatocytes express unusually high levels of uPA, which is thought to induce severe damage in the replicative ability of m-hepatocytes through the activation of plasminogen, fibrinogen, and other proteins within the rough endoplasmic reticulum (RER) involved in proteolysis that lead to functional defects of the RER[21]. In addition, uPA is secreted from m-hepatocytes into the plasma[10], indicating that it circulates to liver tissues through sinusoidal capillaries and activates the conversion of blood plasminogen to plasmin. Therefore, the host liver tissue may provide h-GCCs with a pro-metastatic-like microenvironment. In fact, previous studies have indicated that uPA and its receptor (uPAR) play critical roles in the extravasation of tumors[22-24]. Therefore, the injected h-GCCs are prone to extravasate liver tissues through the portal vein and sinusoid because of the uPA-induced fragility of vascular and sinusoidal endothelia and subsequently engraft liver tissues through an affinity for c-Met. Once the h-GCCs invade liver tissues, they can relatively easily propagate due to c-Met signaling in the host parenchyma, and can consequently replace m-hepatocytes as a result of the uPA-mediated damage. These conditions are also convenient for engraftment and proliferation of normal, healthy h-hepatocytes, as shown in this study when co-transplanted with h-GCCs.

The co-transplantation of h-hepatocytes with h-GCCs also resulted in the development of metastatic colonies in the mice similar to the transplantation of h-GCCs alone. In this type of transplantation experiment, large variances in serum concentrations of replacement marker proteins (h-Alb and h-AFP) were observed. The h-AFP kinetic curves were different from those of h-Alb and exhibited an increase of the serum level through "three steps": initial increase, followed by a plateau or decline, and then a sharp increase. This complex h-AFP kinetic pattern suggests the presence of interactions between the invading cancer cells and the accepting host cells. There seemed to be two groups of animals within the experimental groups, one that more easily accepted xenogeneic cells and another that demonstrated resistance. However, we have consistently observed similar variances in h-Alb levels among individual mice when we generated h-hepatocyte chimeric mice[12], though inbred mice were used as hosts. These variances are accidental in nature and might originate from some differences in manipulation procedures for transplantation as well as uncontrollable differences in the phenotypes of the uPA Tg mice[10]. Despite these variances at the individual level, experimental group B of this study clearly demonstrated that we were able to reproducibly create mice whose livers were co-repopulated with healthy, normal h-hepatocytes and h-GCCs. Both h-hepatocytes and h-GCCs have high affinities for liver tissue, which drives engraftment of the liver and results in the generation of a humanized liver with metastatic cancer cells. We also found that the $RI_{h\text{-}hepatocyte}$ (66.0% ± 12.3%) was significantly higher than $MI_{h\text{-}GCC}$ (12.0% ± 6.8%), which may be a reflection of the difference in the inherent replication rates of the cells and adaptability to the host liver tissues. Our results indicate that h-hepatocytes are, as a whole, superior to h-GCCs in colony growth.

Relevant and reproducible animal models are indispensable tools for deducing the mechanisms of liver metastasis and pharmacokinetics of anti-cancer drugs, and several models have been developed to meet these practical needs, though they are quite limited[2,25-30]. Preclinical tests of anti-cancer drugs for their effectiveness and toxicity in relevant animal models are required prior to application in humans[31]. Toxicity data from non-primate species have been quite poor at predicting outcomes in subsequent human clinical trials, since there are significant differences in the metabolic activities of the hepatocytes between humans and rodent[32-34]. Therefore, animal models with a humanized liver are more physiologic and will provide better tools for analyzing the pharmacokinetics of anti-cancer drugs as well as studying cancer metastasis[35-37]. To our knowledge, no intrahepatic metastatic cancer model with a humanized liver has been available to date[25,30,35-37]. The m-liver in the present study was chimeric and was composed of normal h-hepatocytes and m-hepatocytes. Previous studies have reported that the h-hepatocytes in these chimeric livers are functional and secreted a variety of hepatic proteins, such as Alb, -1 antitrypsin, apolipoprotein A, apolipoprotein E, several clotting factors, and complement proteins present in h-plasma[38]. Transplanted h-hepatocytes also retain normal pharmacological responses, which makes the chimeric mouse model useful for studying the metabolism of compounds that cannot be easily administered to healthy volunteers[14,15]. *In vivo* studies using these mice showed their utility in evaluating the metabolism of drugs catalyzed by both phase I and phase II enzymes[13-15,39,40]. Since the liver functions of

the chimeric mice described in this study have not yet been characterized, future studies are needed to assess the model for anti-cancer drug testing. Taking together, the h-hepatocyte-chimeric mice may provide a useful bridge for studying human liver-related diseases because of the similarities with humans in physiological function and drug kinetics.

In conclusion, we have established a unique and novel animal model for studying liver cancer metastasis. The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for *in vivo* testing of the efficacy and human-type metabolisms of candidate drugs for anti-cancer treatment as well as studying the mechanisms of liver cancer metastasis.

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COMMENTS

Background

One of the major target organs for cancer metastasis is the liver, and therefore, there has been increasing needs for animal models that can sufficiently mimic the pathophysiological situation in human liver and that are suitable for investigating the mechanisms of hepatic cancer metastasis.

Research frontiers

An ideal animal model for liver metastasis of human cancer cells should possess at least two key features. First, the transplanted cancer cells need to invade and colonize the liver of the host. Second, the liver of the host model has to provide the human cells with appropriate pathophysiological microenvironments that recapitulate the human liver *in vivo*. In the present study, the authors established a unique and novel animal model with both of these features.

Innovations and breakthroughs

A liver-humanized mouse was generated by transplanting healthy and normal h-hepatocytes into urokinase type plasminogen activator/severe combined immunodeficient (uPA/SCID) mice (immuno- and liver- compromized mice), and the liver was stably and reproducibly replaced with human hepatocytes. This is the first report of a novel experimental model that sufficiently mimics the pathophysiological situation of human liver.

Applications

The chimeric liver of the uPA/SCID mouse containing both human cancer cells and hepatocytes could be utilized as an appropriate model for the *in vivo* testing of anti-cancer drugs as well as studying the mechanisms of liver cancer metastasis.

Terminology

The uPA/SCID mouse is a transgenic mouse line that expressed uPA under the control of the albumin enhancer/promoter which constitutively damages the hepatocytes due to constant exposure to uPA. A liver- humanized mouse (chimeric mouse) was generated by transplanting healthy and normal human hepatocytes into mouse liver of the uPA/SCID mouse (immuno- and liver-compromized mouse), which had been generated by mating the uPA-Tg mouse with the SCID mouse. This mouse model sufficiently mimics the pathophysiological situation in human liver.

Peer review

This study tries to establish an animal model with h-hepatocyte-repopulated liver for *in vivo* study of liver cancer using uPA/SCID mouse, which could be useful for studying liver cancer metastasis. The authors transfected uPA/SCID mouse either with human gastric cancer cells (h-GCCs) or h-GCCs with h-hepatocytes and observed that both colonies can repopulate mouse liver. The study is well conducted, the manuscript is well-written and the figures are of good quality.

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BRIEF ARTICLE

Endoscopic ultrasound-guided fine needle aspiration in the differentiation of type 1 and type 2 autoimmune pancreatitis

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Abstract

AIM: To investigate the usefulness of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) in the differentiation of autoimmune pancreatitis (AIP).

METHODS: We retrospectively reviewed 47 of 56 AIP patients who underwent EUS-FNA and met the Asian diagnostic criteria. On 47 EUS-FNA specimens, we evaluated the presence of adequate material and characteristic features of lymphoplasmacytic sclerosing pancreatitis (LPSP) and idiopathic duct-centric pancreatitis (IDCP) mentioned in the International Consensus Diagnostic Criteria and examined if these findings make a contribution to the differential diagnosis of type 1 and type 2 AIP. A disposable 22-gauge needle was used for EUS-FNA.

RESULTS: Adequate specimens including pancreatic tissue for differentiating AIP from cancer were obtained from 43 of 47 patients who underwent EUS-FNA. EUS-FNA was performed from the pancreatic head in 21 cases, which is known to be technically difficult when performed by core biopsy; there was no significant difference in the results compared with pancreatic body-tail. Nine of 47 patients met level 1 findings of LPSP and 5 patients met level 2 findings of LPSP. No one met level 1 findings of IDCP, but 3 patients met level 2 findings of IDCP. Of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP", and 3 cases were diagnosed with "probable type 2 AIP" when considering both the level 2 histological findings and response to steroids.

CONCLUSION: EUS-FNA is useful in the differentiation of type 1 and type 2 AIP, particularly in seronegative cases.

Key words: Autoimmune pancreatitis; Endoscopic ultrasound-guided fine needle aspiration; Idiopathic duct centric pancreatitis; Lymphoplasmacytic sclerosing pancreatitis; Pancreatic cancer

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INTRODUCTION

Recently, the International Consensus Diagnostic Criteria (ICDC) for autoimmune pancreatitis (AIP) was proposed by Shimosegawa *et al*[1]. According to these criteria, AIP is classified into 2 types[2]. The histological substance of type 1 AIP is known as lymphoplasmacytic sclerosing pancreatitis (LPSP)[3-6], and type 2 AIP is characterized by a distinct histology called idiopathic duct centric pancreatitis (IDCP)[7-10]. Type 2 AIP patients are generally seronegative and lack other organ involvement (OOI) in contrast to type 1 AIP. However, the absence of serological abnormalities or lack of OOI in patients with AIP does not necessarily imply the diagnosis of type 2, as type 1 also can be seronegative and without OOI. Taking these findings into consideration, ICDC made separate diagnostic criteria for type 1 and type 2 AIP, and histological differentiation is becoming more important for diagnosing AIP.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is now widely accepted as a safe and effective modality for obtaining pancreatic tissue samples[11-14]. There are reports on the usefulness of EUS-FNA in the diagnosis of AIP[15,16] but only negative reports on the differentiation between LPSP and IDCP using specimens obtained by EUS-FNA[17,18]. The findings of expert panel deliberations at the AIP International 2009 Honolulu Meeting reached a uniform consensus that essential histological features of LPSP can only be obtained or evaluated in tissues with preserved architecture, i.e., either a surgical resection specimen or a core biopsy but not FNA[19]. However, a surgically resected specimen can only be obtained from a patient misdiagnosed with pancreatic cancer[20-22], and a core biopsy device may not function properly when used in the duodenum. We thus investigated the usefulness of EUS-FNA in the differentiation of type 1 and type 2 AIP using EUS-FNA with a 22-gauge needle.

MATERIALS AND METHODS

Patients

We retrospectively reviewed 47 patients who underwent EUS-FNA of 56 AIP patients who met the Asian Diagnostic Criteria[23] at our institute between July 2003 and July 2011. Forty-two men and 5 women with a mean age of 62.1 ± 13.6 years (range, 28-86 years) and a mean follow-up period of 839.8 ± 722.7 d (range, 19-2506 d) were included. The mean serum immunoglobulin G4 (IgG4) levels were 626.1 ± 1004.6 mg/dL (range of 4-5850 mg/dL), and 10 patients were seronegative. On 47 EUS-FNA specimens, we evaluated the presence of adequate material and characteristic features of LPSP [lymphoplasmacytic infiltration, storiform fibrosis, obliterative phlebitis, and abundant (> 10 cells per high-power field) IgG4-positive cells] and IDCP [granulocytic infiltration of the duct wall (GEL) or granulocytic acinar infiltrate] mentioned in the histological criteria for ICDC (Table 1). Adequate material indicated an adequate specimen including pancreatic tissue for differentiating AIP from cancer. EUS-FNA was performed from the pancreatic head in 21 cases, an approach that is known to be technically difficult when performed by core biopsy. The histological findings according to the locations of EUS-FNA were also evaluated. Using the results of EUS-FNA, we examined whether these findings make a contribution to the differential diagnosis of type 1 and type 2 AIP. Patients with jaundice or abdominal pain underwent steroid therapy with oral prednisolone (PSL). The initial dose of PSL was 30-40 mg/d, and it was tapered down to the maintenance dose (2.5-5 mg/d) within 12 wk. Relapse was defined as exacerbation of the pancreatic lesion or OOI morphology or emergence of new OOI. OOI include cholangitis[24] [proximal (hilar/intrahepatic) or proximal and distal bile stricture], sialadenitis, nephritis[25], inflammatory bowel disease (IBD), and retroperitoneal fibrosis.

EUS-FNA

After receiving written informed consent, the patients were submitted to conscious sedation with intravenous diazepam under appropriate cardiorespiratory monitoring. EUS-FNA was performed by expert endosonographers with experience of more than five thousand EUS cases. The apparatus used was a convex-type EUS, GF-UCT 240 (OLYMPUS Co., Ltd., Tokyo, Japan) and Prosound α10 (ALOKA Co., Ltd., Tokyo, Japan) with a frequency of 7.5 MHz. The needle used for EUS-FNA was a disposable 22-gauge needle (EZ shot; OLYMPUS Co., Ltd., Tokyo, Japan). After detailed evaluation of the pancreas with the B-mode and confirmation that no vessels were present in the puncture route in the color Doppler mode, EUS-FNA was performed from the stomach to puncture the pancreatic body or tail and from the duodenum to puncture the pancreatic head.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, United States). The χ^2 test and Fisher's exact test were used to compare categorical parameters between the groups. Continuous parameters were presented as the mean ± SD and/or median (range), and Student's *t* test was used. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

EUS-FNA

The number of FNA passes ranged from 1 to 4 with a

Table 1 Histological criteria for International Consensus Diagnostic Criteria

	Level 1	Level 2
Type 1 AIP		
Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells	LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells
Type 2 AIP		
Histology of the pancreas (core biopsy/resection)	IDCP Both of the following: (1) GEL with or without granulocytic acinar inflammation (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltrate (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosign pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; GEL: Granulocytic infiltration of the duct wall; IgG4: Immunoglobulin G4; HPF: High power field.

Table 2 Results of endoscopic ultrasound-guided fine needle aspiration specimen *n* (%)

	Pancreatic head (*n* = 21)	Pancreatic body-tail (*n* = 26)	Total (*n* = 47)	*P* value
Average number of FNA passes	2.00 ± 0.43 (1-3)	2.04 ± 0.514 (1-4)	2.02 ± 0.48 (1-4)	0.78
Adequate sample material	17 (80.9)	26 (100)	43 (91.4)	0.07
Lymphoplasmacytic infiltration	6 (28.6)	10 (38.4)	16 (34.0)	0.68
Storiform fibrosis	12 (57.1)	22 (84.6)	34 (72.3)	0.07
Obliterative phlebitis	0 (0)	0 (0)	0 (0)	1
Abundant IgG4-positive plasmacyte infiltration	3/10 (30)	7/18 (38.8)	10/28 (35.7)	1
Granulocytic infiltration of duct wall	0 (0)	0 (0)	0 (0)	1
Granulocytic acinar infiltrate	1 (4)	2 (7.7)	3 (6.3)	1
Complications	0 (0)	0 (0)	0 (0)	1

IgG4: Immunoglobulin G4; FNA: Fine needle aspiration.

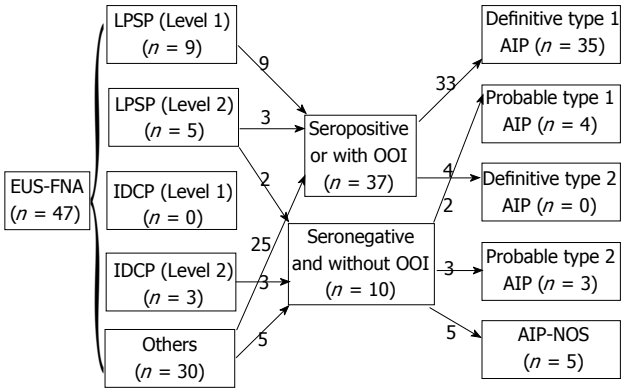

Figure 1 Comparison of endoscopic ultrasound-guided fine needle aspiration with International Consensus Diagnostic Criteria. EUS-FNA: Endoscopic ultra-sound-guided fine needle aspiration; LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; OOI: Other organ involvement; AIP: Autoimmune pancreatitis; NOS: Not otherwise specified.

mean of 2.00 ± 0.48. One pass included approximately 15 to 20 back-and-forth movements in the target lesions. Adequate sample material was obtained from 43 of 47 patients who underwent EUS-FNA as well as 17 of 21 cases from the pancreatic head and all 26 cases from the body and tail. Sixteen of 47 EUS-FNA specimens showed lymphoplasmacytic infiltration, and 34 showed storiform fibrosis, but obliterative phlebitis could not be detected in any of the cases. Abundant IgG4-positive plasmacyte infiltration was shown in 10 of 28 patients who underwent immunostaining. Although GEL was not detected in any of the cases, three cases showed granulocytic acinar infiltrate. No significance was seen in the results of EUS-FNA between those performed at the pancreatic head and those obtained at the body-tail. There were no complications from EUS-FNA (Table 2).

On comparing the histological results of EUS-FNA against ICDC (Figure 1), 9 of 47 patients met level 1 findings of LPSP (Figure 2), and 5 patients met level 2 findings of LPSP. Two of 5 patients who met level 2 findings of LPSP were seronegative and without OOI and were finally diagnosed with "definitive type 1 AIP" after considering both the level 2 histological findings and response to steroids (Table 3). No one met level 1 findings of IDCP (GEL), but 3 patients met level 2 findings of IDCP. All 3 patients were relatively young, seronegative, and had no OOI, including IBD. They were diagnosed with "probable type 2 AIP" (Figure 3) after considering the level 2 histological findings and response to steroids. They have shown improvement without relapse on radiological findings following steroid therapy thus far (Table 4).

DISCUSSION

EUS-FNA is an established and widely used technique to evaluate pancreatic masses. The diagnostic accuracy of EUS-FNA for pancreatic cancer is reported to be between 60% and 90%[26-28], but conclusive diagnosis of

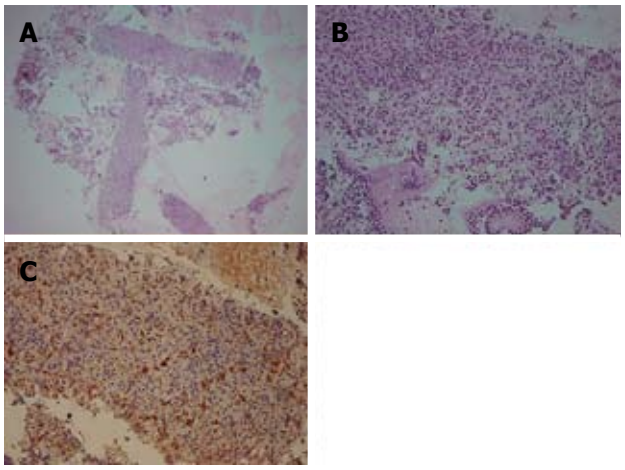

Figure 2 Endoscopic ultrasound-guided fine needle aspiration specimen of "definitive type 1 autoimmune pancreatitis". A, B: Hematoxylin and eosin staining of a resected pancreas specimen obtained by endoscopic ultrasound-guided fine needle aspiration shows replacement of the acinar structure by lymphoplasmacytic infiltration and fibrosis; C: Numerous plasma cells show positive immunoreactivity for immunoglobulin G4.

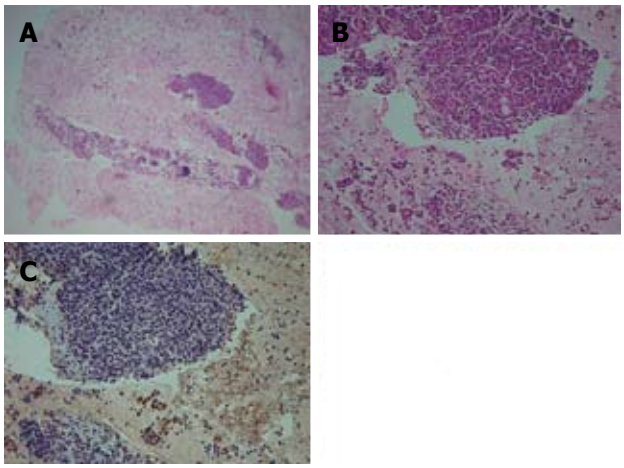

Figure 3 Endoscopic ultrasound-guided fine needle aspiration specimen of "probable type 2 autoimmune pancreatitis". A, B: Hematoxylin and eosin staining of a resected pancreas specimen obtained by endoscopic ultrasound-guided fine needle aspiration shows the infiltration of neutrophils in addition to lymphocyte infiltration and fibrosis; C: Immunostaining for immunoglobulin G4 is negative.

Table 3 Patients with level 2 histological findings of lymphoplasmacytic sclerosing pancreatitis

Case	Sex	Age, yr	IgG4 (mg/dL)	Location	Response to steroid	OOI	Diagnosis
1	Male	74	263	Diffuse	(+)	Nephritis	Definitive type 1 AIP
2	Female	71	364	Diffuse	(+)	Cholangitis	Definitive type 1 AIP
3	Male	54	230	Focal	(+)	Sialadenitis	Definitive type 1 AIP
4	Male	47	104	Focal	(+)	None	Definitive type 1 AIP
5	Male	57	46	Focal	(+)	None	Definitive type 1 AIP

IgG4: Immunoglobulin G4; OOI: Other organ involvement; AIP: Autoimmune pancreatitis.

Table 4 Patients with level 2 histological findings of idiopathic duct-centric pancreatitis

Case	Sex	Age, yr	IgG4 (mg/dL)	Location	Response to steroid	OOI	Follow-up, d	Relapse	Diagnosis
1	Male	28	69	Diffuse	(+)	(-)	973	(-)	Probable type 2 AIP
2	Female	31	43	Diffuse	(+)	(-)	425	(-)	Probable type 2 AIP
3	Male	30	23	Focal	(+)	(-)	120	(-)	Probable type 2 AIP

IgG4: Immunoglobulin G4; OOI: Other organ involvement; AIP: Autoimmune pancreatitis.

AIP is often difficult due to the small size of specimens obtained by FNA. Recently, there have been several reports on the usefulness of EUS-guided tru-cut biopsy (EUS-TCB) for the diagnosis of AIP[29-31]. Tru-cut biopsy needles have been developed to acquire samples while preserving tissue architecture, thus allowing histological examination[32,33]. Previous reports describe the safety and the technical feasibility of performing EUS-TCB from a transgastric approach. However, the TCB device may not function properly when used in the second portion of the duodenum, and there is also some difficulty when using the TCB device from the duodenal bulb and along the greater curvature of the antrum[29,34]. Moreover, because a 19-gauge needle is used for EUS-TCB, the risk of bleeding is higher compared with EUS-FNA using a 22-gauge needle, indicating that reexamination of safety is required. We previously reported[35,36] the feasibility of EUS-FNA using a 22-gauge needle for the histological evaluation of gastrointestinal submucosal tumors, and we believe that this method can also be applied to pancreatic lesions. In our study, adequate material for differentiating cancer from AIP was obtained in 43 of 47 cases (91.4%), and no significant difference in EUS-FNA results was seen between those obtained from the pancreatic head and body-tail. Nine of 47 patients (19.1%) met 3 of 4 characteristic features of LPSP and were diagnosed with "definitive type 1 AIP" based on histological findings alone. Detailed analysis of 8 patients who showed level 2 histological findings of type 1 or type 2 AIP revealed that 3 patients with level 2 findings of type 1 were seropositive and/or with OOI and could be diagnosed with "definitive type 1 AIP" without histological findings, but the other 5 patients were seronegative and without OOI and diagnosed with "definitive type 1 AIP" or "probable type 2 AIP" based on combination of the level 2 histological findings and the response to steroid treatment. Therefore, out of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP", and 3 cases were diagnosed with "probable type 2 AIP" using the histological findings of EUS-FNA. As mentioned earlier, type 1 AIP often can be di-

agnosed without histology, but it is difficult to differentiate type 1 and type 2 AIP when results are seronegative and without OOI. We believe histological evaluation of EUS-FNA is rather important in such cases.

In conclusion, EUS-FNA is useful in diagnosing AIP even when performed from the pancreatic head and may also provide complementary histological information to distinguish type 1 and type 2 AIP, particularly in seronegative cases.

COMMENTS

Background

Recently, the International Consensus Diagnostic Criteria (ICDC) for autoimmune pancreatitis (AIP) was proposed. ICDC made separate diagnostic criteria for type 1 and type 2 AIP, and histological differentiation is becoming more important for diagnosing AIP. There have been reports on the usefulness of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) in the diagnosis of AIP but only negative reports on the differentiation between type 1 and type 2 AIP using specimens obtained by EUS-FNA.

Research frontiers

In the area of AIP, the research hotspot is how to obtain sufficient materials from AIP patients and differentiate type 1 and type 2 AIP correctly.

Innovations and breakthroughs

Adequate specimens including pancreatic tissue for differentiating AIP from cancer were obtained from 43 of 47 patients who underwent EUS-FNA. EUS-FNA was performed from the pancreatic head in 21 cases, which is known to be technically difficult when performed by core biopsy; there was no significant difference in the results compared with pancreatic body-tail. Of 10 seronegative cases, 2 cases were diagnosed with "definitive type 1 AIP," and 3 cases were diagnosed with "probable type 2 AIP" when considering both the level 2 histological findings and response to steroids.

Applications

The study results suggested that EUS-FNA (instead of core biopsy) was useful in diagnosing AIP even when performed from the pancreatic head and may also provide complementary histological information to distinguish type 1 and type 2 AIP, particularly in seronegative cases.

Terminology

Type 1 and type 2 AIP: The histological substance of type 1 AIP is known as lymphoplasmacytic sclerosing pancreatitis, and type 2 AIP is characterized by a distinct histology called idiopathic duct centric pancreatitis. Type 2 AIP patients are generally seronegative and lack other organ involvement (OOI) in contrast to type 1 AIP. However, the absence of serological abnormalities or lack of OOI in patients with AIP does not necessarily imply the diagnosis of type 2, as type 1 also can be seronegative and without OOI.

Peer review

The authors reported the usefulness of EUS-FNA in the diagnosis of type 1 and type 2 AIP and also stressed the importance of this method for the differential diagnosis between AIP and pancreatic cancer especially in the cases with negative results of serology and absence of other organ involvement. The content is clear and the discussion is straightforward. This paper is useful for understanding the ICDC and the classification of type 1 and 2 AIP.

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BRIEF ARTICLE

Non-invasive determination of hepatic steatosis by acoustic structure quantification from ultrasound echo amplitude

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Abstract

AIM: To use leptin-deficient (*ob/ob*) mice with demonstrated differences in steatosis levels to test a new diagnostic method using the acoustical structure quantification (ASQ) mode and the associated analytical parameter, "focal disturbance ratio" (FD-ratio).

METHODS: Nine *ob/ob* mice, at 5, 8, and 12 wk of age (n = 3 in each age group), were used as models for hepatic steatosis. Echo signals obtained from ultrasonography in the mice were analyzed by ASQ, which uses a statistical analysis of echo amplitude to estimate inhomogeneity in the diagnostic region. FD-ratio, as calculated from this analysis, was the focus of the present study. FD-ratio and fat droplet areas and sizes were compared between age groups.

RESULTS: No fibrosis or inflammation was observed in any of the groups. The fat droplet area significantly (P < 0.01) increased with age from 1.25% ± 0.28% at 5 wk to 31.07% ± 0.48% at 8 wk to 51.69% ± 3.19% at 12 wk. The median fat droplet size also significantly (P < 0.01) increased with age, from 1.33 (0.55-10.52) μm at 5 wk, 2.82 (0.61-44.13) μm at 8 wk and 6.34 (0.66-81.83) μm at 12 wk. The mean FD-ratio was 0.42 ± 0.11 at 5 wk, 0.11 ± 0.05 at 8 wk, and 0.03 ± 0.02 at 12 wk. The FD-ratio was significantly lower at 12 wk than at 5 wk and 8 wk (P < 0.01). A significant negative correlation was observed between the FD-ratio and either the fat droplet area (r = -0.7211, P = 0.0017) or fat droplet size (r = -0.9811, P = 0.0052).

CONCLUSION: This tool for statistical analysis of signals from ultrasonography using the FD-ratio can be used to accurately quantify fat *in vivo* in an animal model of hepatic steatosis, and may serve as a quantitative biomarker of hepatic steatosis.

Key words: Non-alcoholic fatty liver disease; Quantitation of hepatic steatosis; Animal model; Focal disturbance ratio; Acoustic structure quantification; Ultrasonography

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinically important disease that occurs in subjects with underlying conditions such as obesity or insulin resistance, and is frequently accompanied by metabolic syndrome, including diabetes, hyperlipidemia and/or hypertension[1-5]. Non-alcoholic steatohepatitis (NASH) is the most extreme form of NAFLD, and is regarded as a major cause of cirrhosis of the liver of unknown cause[6-10]. Methods for early detection and assessment of NAFLD through quantitative measurement of steatosis are needed to achieve earlier intervention and avoid the progression to cirrhosis.

The gold standard for quantitative assessment of steatosis has been considered to be liver histology[11]. However, liver biopsy shows various limitations, such as potential sampling error, difficulties repeating the procedure because of ethical concerns, and complications including bleeding[12]. Non-invasive alternatives to liver biopsy thus need to be established.

Several methods have recently been established for non-invasively quantifying steatosis using imaging techniques. Non-invasive modalities such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) have been employed for the assessment of hepatic steatosis[13-15]. However, using these modalities for the repeated evaluation of NAFLD is difficult, because CT involves radiation exposure and MRI is expensive to perform. From these perspectives, ultrasonography represents an excellent examination modality that is minimally invasive, inexpensive, and can be performed repeatedly with no risk to the patient. Furthermore, ultrasonography is known to be highly sensitive for detecting fat accumulation in the liver[16]. However, the diagnostic performance of ultrasonography depends on the empirical and qualitative reading skills of the examiner, and quantitative methods to evaluate liver lipogenesis have yet to be established.

In recent years, Yamaguchi *et al*[17,18] have reported that diffuse pathological changes in the liver tissue can be quantitatively evaluated based on the statistical deviation of ultrasound signals compared to normal liver. Generally, ultrasonographic images of parenchymal organs such as the liver are designated as having a "speckle pattern" consisting of numerous fine echo spots. The speckle pattern is constructed by ultrasonic interference of scattered ultrasound waves generated by innumerable reflexive objects that are distributed closer than the ultrasonic wavelength[17-19]. Image analysis of speckle patterns has been used to identify tissue characteristics associated with chronic liver diseases, because the pattern changes according to the structural characteristics of the medium. One such analytical method, the probability density function (PDF) of the echo amplitude of a speckle pattern, has been reported to be approximated by a function called the Reyleigh distribution. Moreover, Toyoda *et al*[20] proposed the acoustical structure quantification (ASQ) method and reported the possibility of quantifying diffuse liver disease or monitoring regression/progression in cases of liver fibrosis and during treatment. However, no reports have yet described assessments of hepatic steatosis in NAFLD patients using this tool for the statistical analysis of ultrasonic signals.

The present study aimed to validate a quantitative imaging technique used to detect and measure steatosis with statistical information from ultrasound echo signals with the focal disturbance ratio (FD-ratio) as a parameter in leptin-deficient (*ob/ob*) mice, a pure NAFLD model.

MATERIALS AND METHODS

This study was a collaborative effort between Iwate Medical University and Toshiba Medical Systems. However, no direct financial support was received from Toshiba Medical Systems for this study.

Animals

The animal research protocols for this prospective study were approved by our institutional research animal resource center. Nine 5 wk old (at the start of the study) male *ob/ob* mice were purchased from Charles River Laboratories (Yokohama, Japan) and maintained on conventional food and water throughout the experiment. These mice were divided into 3 groups (n = 3 each) that underwent the experiment described below at 5, 8 and 12 wk old, respectively.

General anesthesia was induced in mice by intraperitoneal administration of 40-50 mg/kg pentobarbital sodium (Ovation Pharmaceutical, Deerfield, IL), and underwent laparotomic ultrasonography, prior to having the liver extracted for histological examination.

Ultrasonographic imaging

An AplioXG ultrasound scanner (Toshiba Medical Systems, Otawara, Japan) was combined with a 12 MHz linear transducer (PLT-1204BT) for ultrasonographic investigations in this study. The scan mode was harmonic B-mode imaging (T: 6.0/R: 12.0 MHz). Display depth and transmit focus were fixed at 15 mm and 7.5 mm, respectively, and cross-sectional images of the hepatic parenchyma were recorded digitally with raw data, consisting substantially of the linear amplitudes without any cosmetic image processing. Raw data were uploaded to a personal computer in the DICOM format. FD-ratio was determined using ASQ software (details described in the next section). A region of interest (ROI) was set to a fixed depth of 2.5 mm to the liver surface (Figure 1). FD-ratio was measured 10 times in succession, and the mean value (after excluding outliers) was used as the final result. In parallel, mean echo intensity of the hepatic parenchyma was measured using Image-J image analysis software (NIH, United States).

Analytical method

The principles of the ASQ method[20] are as follows. When echo signals are generated from very small, dense scatters located beyond the limit of spatial resolution, the pattern of the ultrasound image is constructed based on the interference of the sound waves (speckle noise). In that

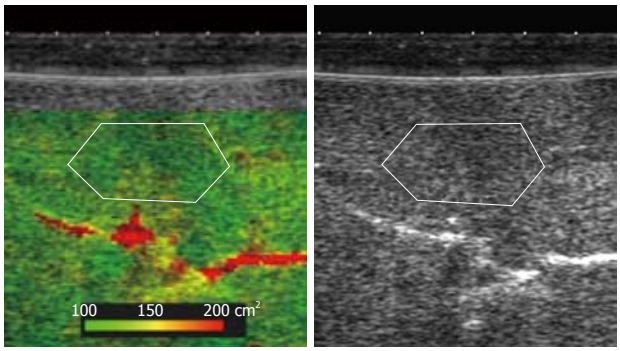

Figure 1 Acoustic structure quantification imaging. In parametric imaging, the intensity distribution can be visualized and displayed on a split screen by color coding. The large-region of interest was set at a fixed depth of 2.5 mm from the liver surface.

case, the PDF of the echo amplitude can be approximated using the Rayleigh distribution function[21]. In normal liver parenchyma, such statistical results are not described by the Rayleigh distribution due to the existence of structures such as vessel walls. Results for livers with either nodules or fibrosis, i.e., liver cirrhosis, are even less similar to the Rayleigh distribution. We hypothesized that in the case of progression of fat drops, these scatters would generate wave interference or mask the original small structures, which would change the PDF to more closely resemble a Rayleigh distribution.

Once the examiner sets a comprehensive ROI (hereinafter referred to as a large-ROI) on the image, several hundred small ROIs (small-ROIs hereinafter) are automatically set therein to calculate the PDF (Figure 2A). The essential parameter, C_m^2, in the analysis is defined by the equation:

$$C_m^2 = \frac{\sigma_m^2}{\sigma_R^2(\mu_m)} = \left(\frac{\pi}{4-\pi}\right)\frac{\sigma_m^2}{\mu_m^2}$$

where μ and σ^2 are the average and variance of the echo amplitude in a small-ROI, respectively. The $\sigma_R^2(\mu)$ is a variant if the Rayleigh distribution is estimated from the measured average. Multiple results for small-ROIs in a large-ROI are displayed as an occurrence histogram of C^2 (real line in Figure 2B). If samples consist of speckle noise, the C^2 histogram will gather to 100 with narrow variance, while structural information will make the average value larger and variance wider.

FD-ratio is calculated in the following manner. First, C_m^2 is defined as:

$$C_m^2 = \frac{\sigma_m^2}{\sigma_R^2(\mu)} = \left(\frac{\pi}{4-\pi}\right)\frac{\sigma_m^2}{\mu^2}$$

where σ_m is the variance calculated from limited samples less than $\mu + 4\sigma$. If the ratio C^2/C_m^2 is larger than the threshold α, the result of C_m^2 is eliminated from the histogram (real line), but added to the alternative histogram (dotted line). The FD-ratio is the ratio of the area under the curve (AUC) for these two histograms: R_{FD} = [AUC (real)]/[AUC (dotted)].

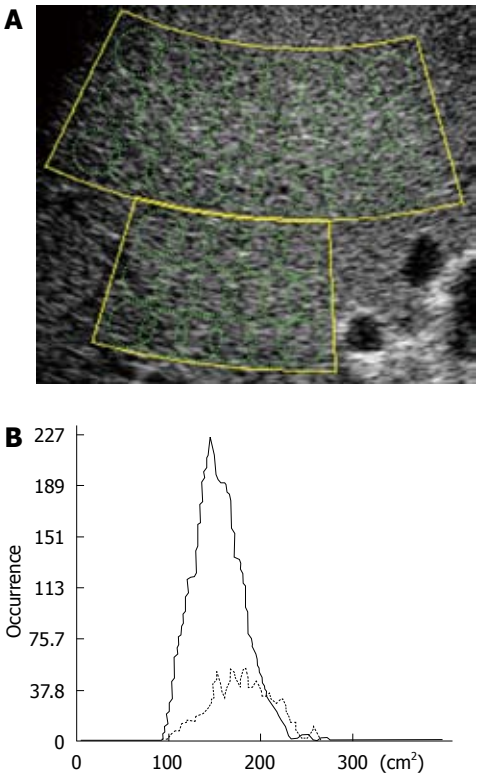

Figure 2 Schematic of region of interests used for statistical analysis of the radio frequency signal with the acoustic structure quantification method, and the C_m^2-histogram. A: The set large-region of interest (ROI) actually consists of several hundred small ROIs used to calculate multiple C_m^2 (intensity or amplitude) values; B: Results are shown as the occurrence in the C_m^2 histogram.

Here, the threshold α was set to 1.2, so $R_{FD} = 0$ when the samples show a Rayleigh distribution for the PDF, and has a positive value in the presence of tiny structural changes.

The ASQ software also has an imaging function that reconstructs the 2-dimensional color map of the C^2 measured for each position (parametric image).

Histopathological analysis

Histopathological examinations were performed by an experienced pathologist certified by the Japanese Society of Pathology. Images from liver biopsy samples were recorded using the JPEG format. Using Image-J software, the percentage fat area was calculated from the ratio between total fat tissue area and total specimen area, and the mean value was used to denote the fat droplet area. In addition, again using Image-J software, the maximum diameter of fat droplets in 8 and 12 wk old mice was measured, and median values were used to denote the size of fat droplets. The fat droplet area and size obtained in this manner were compared with the FD-ratio.

Statistical analysis

Values are shown as mean ± SD, or median (range) according to the distribution of values. Stat View software (version 5.0; SAS Institute, Cary, NC, United States) was used for all statistical analysis. The Spearman rank-order

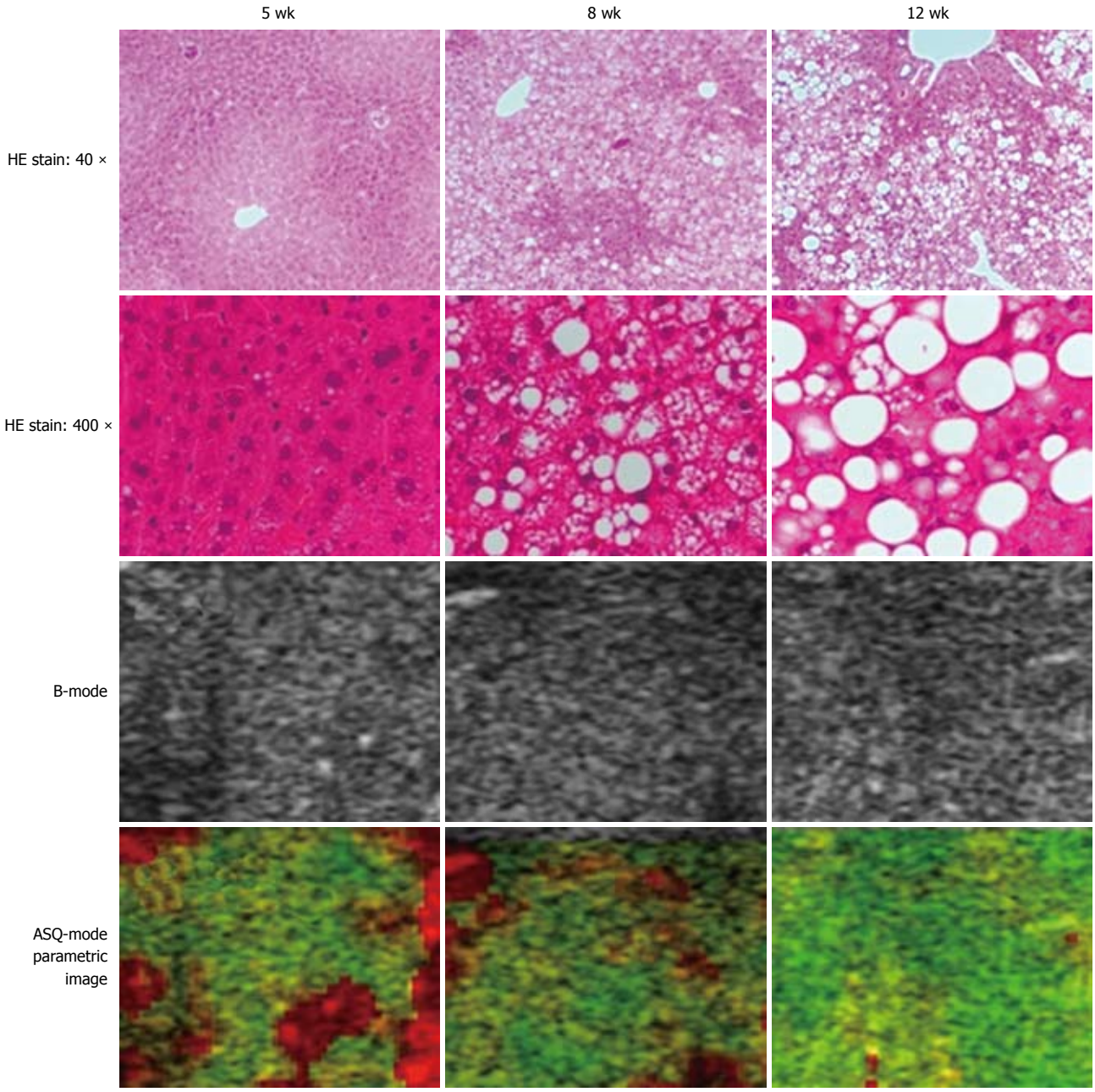

Figure 3 Representative histological findings, B-mode images and acoustic structure quantification-mode images for each group. No fibrosis or inflammation was observed in any groups. Large fat droplets were mainly observed at 12 wk old. In parametric imaging, red and green existed together at 5 wk, and red decreased and green was more abundant at 12 wk. ASQ: Acoustical structure quantification.

correlation test was used to study correlations between two variables, with a significant correlation considered to exist for values of $P < 0.05$, and for correlation coefficient $r \geqslant 0.40$. The Tukey-Kramer method was used for multiple comparison tests, and values of $P < 0.05$ were considered to indicate a significant difference.

RESULTS

Comparison of histological findings and liver echogenicity

No fibrosis or inflammation was observed in any groups. Large fat droplets were mainly observed at 12 wk (Figure 3). Fat droplet area increased significantly ($P < 0.01$ each) with age from 1.25% ± 0.28% at 5 wk to 31.07% ± 0.48% at 8 wk, and to 51.69% ± 3.19% at 12 wk. Median fat droplet size also increased significantly ($P < 0.01$ each) with age, from 1.33 μm (range: 0.55-10.52 μm) at 5 wk to 2.82 μm (range: 0.61-44.13 μm) at 8 wk and 6.34 μm (range: 0.66-81.83 μm) at 12 wk (Figure 4). Mean Gray values in each group were 65.31 ± 22.52 at 5 wk, 65.95 ± 19.41 at 8 wk and 91.32 ± 21.83 at 12 wk (Figure 5). Although no differences were observed between the 5 and 8 wk old groups, mean gray value was significantly elevated in the 12 wk old group, with an increase observed in the brightness of the hepatic parenchyma.

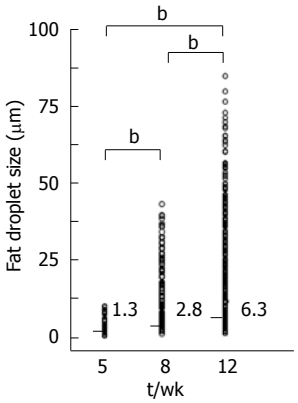

Figure 4 Comparison of fat droplet size. Median fat droplet size increased significantly ($^bP < 0.01$) with age, from 1.33 μm (range: 0.55-10.52 μm) at 5 wk, to 2.82 μm (range: 0.61-44.13 μm) at 8 wk and 6.34 μm (range: 0.66-81.83 μm) at 12 wk.

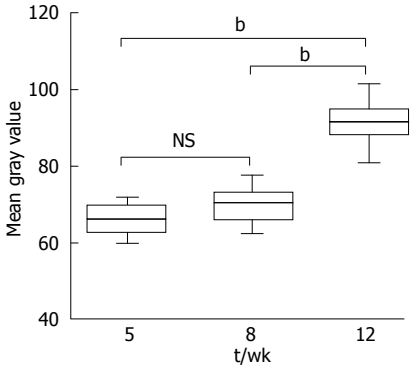

Figure 5 Comparison of liver echogenicity. Mean gray values in each group were 65.31 ± 22.52 at 5 wk, 65.95 ± 19.41 at 8 wk, and 91.32 ± 21.83 at 12 wk. Although no difference was apparent between the 5 and 8 wk old groups, mean gray value was significantly elevated in the 12 wk old group, with increased brightness of the hepatic parenchyma. $^bP < 0.01$. NS: Not significant.

Relationship between FD-ratio and fat droplet area or size

Mean FD-ratio was 0.42 ± 0.11 at 5 wk, 0.11 ± 0.05 at 8 wk, and 0.03 ± 0.02 at 12 wk. The FD-ratio was significantly lower at 12 wk than at 5 or 8 wk ($P < 0.01$ each). In parametric imaging, red and green existed together at 5 wk, and the red had decreased and green was more abundant at 12 wk (Figure 3). A significant negative correlation was observed between FD-ratio and both fat droplet area ($r = -0.7211$, $P = 0.0017$) (Figure 6A) and fat droplet size ($r = -0.9811$, $P = 0.0052$) (Figure 6B).

DISCUSSION

The results of this study demonstrated a close correlation between FD-ratio and the degree of histologically evaluated fat accumulation in the liver. These data suggest that ASQ analysis of liver ultrasonography and its representative parameter, FD-ratio, may offer a reliable new clinical modality for the assessment of liver steatosis.

With regard to the ultrasonographic diagnosis of fatty liver disease, Joseph *et al*[16] advocated the bright liver pattern, and attempts to quantify the hepatosplenic contrast or hepatorenal contrast were subsequently proposed[22-24]. Although findings of a bright liver pattern and hepatorenal contrast are widely accepted as sensitive, reliable findings for the presence of fatty liver disease, no quantitative method to evaluate the severity of hepatic fat accumulation has previously been established. The difficulties in ultrasonographic diagnosis may originate from the fact that image information obtained by conventional ultrasonography lacks an objective or quantitative nature, unlike the X-ray absorbance in CT. In contrast, ASQ analysis generate objective data and provide a quantitative assessment of liver histology with respect to fat accumulation. Taking into account the non-invasive and inexpensive nature of ultrasonography, ASQ analysis could become a mainstay in the diagnosis of fatty liver disease and evaluations of disease severity and response to treatment. In addition, these studies of tissue characterization using ASQ analysis could lead to the development of methods for the quantitative diagnosis of other diffuse liver diseases, including fibrosis or inflammation, thus decreasing the need for liver biopsies.

With regard to the ASQ method, procedures are undertaken to analyze the RF signals for each of a large number of small-ROIs set up within a large ROI, for the purpose of improving analytical precision[17-19]. The assumption is that in small-ROIs with a high degree of de-

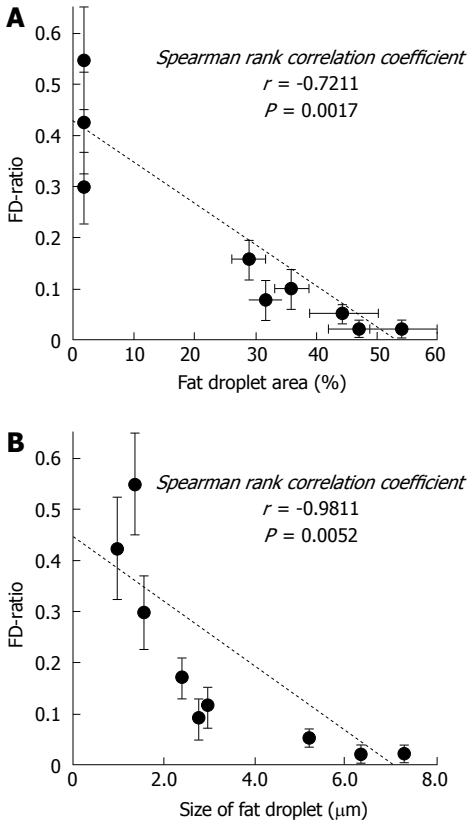

Figure 6 Relationship between "focal disturbance ratio" and fat droplet area or size of fat droplets. A: Significant negative correlations were observed between focal disturbance ratio (FD-ratio) and either fat droplet area ($r = -0.7211$, $P = 0.0017$); B: Fat droplet size ($r = -0.9811$, $P = 0.0052$).

viation from the Rayleigh distribution, the strength of the signals contained therein would be non-homogeneous. We also assumed the presence of two kinds of inhomogeneous samples, i.e., diffuse inhomogeneity and focal inhomogeneity, and by placing our focus on the small-ROIs with a high degree of deviation from the Rayleigh distribution resulting from a focally inhomogeneous structure, we established FD-ratio as a parameter.

Conversely, a bright liver pattern and vascular blurring are observed in fatty livers, due to reflection and scattering of the ultrasound waves and physical pressure on small blood vessels by ballooning hepatocytes induced by the fat droplets. We predicted that, due to the large number of fat droplets assembled densely (and thereby enveloping structures such as small blood vessels and bile ducts), the brightness of the hepatic parenchyma would be increased, and hepatic vein walls would become blurred, thus resulting in homogenization of the signal strength in each small-ROI and a decrease in the number of focally inhomogeneous small-ROIs. As a result, we believed that, as the area and diameter of fat droplets increased, the FD-ratio would decrease. Interestingly, no significant difference was seen in the brightness of hepatic parenchyma between 5 and 8 wk old mice, while FD-ratios were lowest in 8 wk old mice. We can therefore infer that a tool for the statistical analysis of ultrasonic signals may detect, beyond a qualitatively oriented reading ability, small changes in the speckle pattern caused by steatosis.

In conclusion, a novel tool for the statistical analysis of ultrasonic signals using FD-ratio as a parameter would be useful for the quantitative evaluation of liver steatosis. FD-ratio can therefore be used as a non-invasive biological marker for the early detection and quantitative evaluation of hepatic steatosis.

ACKNOWLEDGMENTS

We wish to thank Ms. Yuriko Mikami for her invaluable technical assistance with ultrasonography.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. The gold standard for quantitative assessment of steatosis has been considered to be liver histology. However, liver biopsy shows various limitations and repeating the procedure is difficult because of ethical concerns. Non-invasive alternatives to liver biopsy are thus needed.

Research frontiers

Non-invasive modalities such as ultrasonography, computed tomography, and magnetic resonance imaging have been employed for the assessment of hepatic steatosis. Researchers have recently reported that diffuse pathological changes in liver tissue can be quantitatively evaluated as the statistical deviation of ultrasound signals compared to normal liver. However, no reports have described the assessment of hepatic steatosis in NAFLD patients using this tool for the statistical analysis of ultrasonic signals.

Innovations and breakthroughs

This study validated a quantitative imaging technique used to detect and measure hepatic steatosis with statistical information from ultrasound echo signals using focal disturbance ratio (FD-ratio) as a parameter in an animal model.

Applications

FD-ratio can be used as a non-invasive biological marker for the early detection and quantitative evaluation of hepatic steatosis.

Peer review

The authors present some pilot data from their research on the diagnostic utility of a new ultrasound technique in an animal model of fatty liver.

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BRIEF ARTICLE

Differential roles of EPS8 in carcinogenesis: Loss of protein expression in a subset of colorectal carcinoma and adenoma

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Abstract

AIM: To analyze the epidermal growth factor receptor pathway substrate 8 (EPS8) expression status and role in colorectal carcinogenesis given that EPS8 has a conserved actin barbed-end capping function that is required for proper maturation in intestinal cells.

METHODS: We studied 8 colon cancer cell lines and 58 colorectal tumors (19 adenomas and 39 carcinomas). We performed expression microarray analysis of colon cancer cell lines followed by loss of heterozygosity (LOH) analysis and immunohistochemistry for EPS8 expression in colon tumors. Subsequently, we performed mutation analysis by direct sequencing and methylation analysis by bisulfite sequencing and methylation-specific polymerase chain reaction assays.

RESULTS: Expression microarray analysis of colon cancer cell lines showed overexpression of EPS8 transcript in all lines but RKO. Genome wide loss of heterozygosity (LOH) analysis of colon tumors, showed considerable LOH at the *EPS8* gene locus. Immunohistochemically, EPS8 was constitutively expressed in normal colonic mucosa with a dot-like supranuclear localization with accentuation at the luminal surface supporting its proposed role in epithelial maturation. Nineteen colon tumors (4 adenoma, 15 carcinoma) out of 51 (37%) showed strikingly tumor specific EPS8 protein loss. Of the remaining tumors, 5/51 (2 adenoma, and 3 carcinoma, 10%) showed marked overexpression, while 27/51 tumors (53%) showed retained expression. Mutation analysis revealed a missense mutation (c.794C>T, p.R265C) in exon 8 in RKO. The *EPS8* promoter was also methylated in RKO, but there was no significant methylation in other cell lines or carcinoma specimens.

CONCLUSION: The loss of EPS8 expression in colorectal adenomas and carcinomas suggests that down regulation of this gene contributes to the development of a subset of colorectal cancers, a finding which could have applications in diagnosis and treatment.

Key words: Actin capping; Colon cancer; Epidermal growth factor receptor pathway substrate 8; Hypermethylation; Immunohistochemistry; RKO

Peer reviewer: Dr. Jan Mollenhauer, PhD, Professor, Head of Molecular Oncology, Institute for Medical Biology, University of Southern Denmark, Winsloewparken 25, 5000 Odense C, Denmark

Abdel-Rahman WM, Ruosaari S, Knuutila S, Peltomäki P. Differential roles of EPS8 in carcinogenesis: Loss of protein expression in a subset of colorectal carcinoma and adenoma. *World J Gastroenterol* 2012; 18(29): 3896-3903 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i29/3896.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i29.3896

INTRODUCTION

Epidermal growth factor receptor pathway substrate 8 (EPS8) is a 97-kDa protein that is tyrosine phosphorylated following stimulation of receptor tyrosine kinases (RTK)[1]. EPS8 plays a role in signal transduction from RTK and PI3K[2,3] leading to Rac-mediated actin remodeling, ruffle formation and cell motility[4]. In *Caenorhabditis elegans* (*C. elegans*), *eps-8* knockdown animals were zygotic lethal due to major defects in the gut, and the isoform EPS-8A was shown to be required for proper apical morphogenesis in the intestinal cells. This phenotype was correlated with an actin barbed-end capping activity, which is present in the C terminus of the EPS-8A isoform and is required for coordinately terminated elongation of the microvillar actin bundle core[5]. This function of EPS8 protein is conserved throughout evolution[6].

EPS8 was recently shown to be overexpressed in advanced stage human cancers including colon cancer cell lines and specimens[7]. Our expression microarray analysis of colon cancer cell lines confirmed this overexpression. Interestingly, there was a strikingly low level of *EPS8* in RKO, a colon cancer cell line with a marked lack of constitutive β-catenin regulated transcription[8], which prompted us to conduct a comprehensive immunohistochemical, genetic, and epigenetic analysis of *EPS8* alterations in colorectal cell lines and patient specimens.

MATERIALS AND METHODS

Patients and samples

We studied 8 colon cancer cell lines (RKO, HCA7, KM12, LoVo, DLD1, HCT116, SW48, LIM1215) and 58 colorectal tumors (19 adenomas and 39 carcinomas) of which 21 tumors (4 adenomas and 17 carcinomas) belong to a well characterized series of familial colon cancer type X (FCC-X). 15 adenomas and 22 carcinomas were sporadic. Clinicopathological characteristics of these cohorts are available in our previous publications[9-11]. The FCC-X originated from 19 cancer families clinically indistinguishable from Lynch syndrome (hereditary non-polyposis colon cancer), but screening negative for the known predisposing genes by multiple techniques[12]. We identified distinct molecular features in these tumors including high frequency of genomically stable carcinomas with membranous β-catenin, however, the predisposing defects in these families remain elusive[9]. The sporadic colorectal tumors were selected from a larger cohort with the aim to include equal numbers of tumors with membranous *vs* nuclear β-catenin.

Fresh frozen and/or paraffin derived specimens of tumor and matching normal tissues were collected from pathology departments of different hospitals and used for immunohistochemical analysis and DNA extraction according to standard protocols. All human specimens were obtained after informed consent and approvals from the appropriate institutional review boards of the Helsinki University Central Hospital.

mRNA expression analysis by microarrays

Analyses were performed using HG-U133 Plus 2.0 array (Affymetrix, Santa Clara, CA, United States). The protocols for HG-U133 Plus 2.0 arrays were as described by the manufacturer (Affymetrix, Santa Clara, CA, United States). Briefly, total RNA was extracted from cell lines by RNeasy (Qiagen, Valencia, CA, United States). An aliquot of each RNA sample was run on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) to visualize and quantify the degree of RNA integrity. Double-stranded cDNA was synthesized from 5 μg of total RNA using the GeneChip One-Cycle cDNA synthesis kit, followed by cleanup with the GeneChip Sample Cleanup Module, *in vitro* transcription (IVT) and Biotin labeling reaction using the GeneChip IVT Labeling kit, and clean-up and quantification of the biotin-labeled cRNA yield by spectrophotometric analysis. All kits were from Affymetrix. Fragmentation of the 8 μg cRNA and hybridizations to test chips and the HG-U133 Plus 2.0 array were carried out according to Affymetrix protocols, and microarrays were processed by the Affymetrix Fluidics Station 450 and scanned with an Affymetrix GeneChip Scanner 7G. Captured images were analyzed using Microarray Suite version 5.0 algorithm (Affymetrix). All quality control criteria recommended by Affymetrix were observed in the "Test" chips and sample chips.

The hybridization data were pre-processed using Robust Multi-array Average (RMA[13]), designed to enhance the comparability of expression measures between separate arrays. RMA pre-processing produces a single expression measure for each probe set in the Affymetrix array which can be readily used in subsequent analyses. As duplicate arrays were available for each cell line, the median of the two RMA values was used as the expression value. Gene assignments of the probes were extracted from the Affymetrix annotation files and genes with ambiguous information about the physical location were excluded from the analysis.

EPS8 loss of heterozygosity analysis

For the *EPS8* loss of heterozygosity (LOH) analysis we chose two microsatellite markers spanning the *EPS8* gene locus at Ensembl cytogenetic band 12p12.3 and surrounding the gene from both directions (http://www.ensembl.org). The physical distances between loci in mega-bases according to Ensembl are given in parentheses: pter D12S1580 - (2.4 Mb) - EPS8 - (0.4 Mb) - D12S1728 qter. The polymerase chain reaction (PCR) amplification primers were from Généthon Microsatellite Maps at http://

www.genlink.wustl.edu/genethon_frame. The forward primers were fluorescently labeled with carboxyfluorescein and PCR fragments were run on the ABI3730 sequencer/genotyper and results analyzed using GeneMapper v3 software (Applied Biosystems, Forster City, CA, United States) as described previously[9]. A sample was scored as showing LOH, if one of the alleles had decreased 40% or more, and borderline LOH or allelic imbalance, if the decrease was 25%-39% for one allele.

Microsatellite instability analysis

Microsatellite instability status was determined using the Bethesda panel of 5 microsatellite markers and additional markers as described[9,14,15]. Tumors with two or more unstable markers were considered to have high-degree microsatellite instability (MSI-H), while those with one unstable marker had low-degree microsatellite instability (MSI-L) and those with no unstable markers were microsatellite stable (MSS). MSI-H cancers were mostly excluded from this study cohort to enable the LOH study.

Immunohistochemical analysis

Four-micrometer sections from formalin-fixed paraffin-embedded tissues were mounted on silanized slides (Dako, Glostrup, Denmark) and air-dried overnight at 37 ℃. After de-waxing and re-hydration in distilled water, sections were subject to heat-induced target retrieval in 1 mmol/L ethylenediaminetetraacetic acid buffer pH 8.0 for 5 min at 750 W followed by 5 min at 450 W in a microwave oven. After cooling, the slides were washed in Tris-buffered saline pH 7.2 and subsequent staining steps were performed manually with the Dako EnVision+ System, Peroxidase (DAB), according to the manufacturer's instructions (Dako, Glostrup, Denmark). In addition, after blocking endogenous peroxidase activity, and prior to incubation with the primary antibody, the sections were incubated with 10% normal (non-immune) goat serum (Dako, Glostrup, Denmark) for 30 min. The primary antibodies were purified rabbit polyclonal anti EPS8 Antibody (C-terminal, clone RB4006, Abgent, San Diego, CA, United States) and purified mouse monoclonal anti-β-catenin antibody (clone 14, BD Transduction Laboratories, Erembodegem, Belgium). Paired tumor and normal mucosa were in the same section and the normal tissues were used as an internal reference for evaluation of staining results. β-catenin immunohistochemical staining for identification of its sub-cellular localization and the interpretation of results were performed as described[9]. β-catenin expression was considered aberrant if there was nuclear staining of more than 10% or cytoplasmic staining of more than 50% of tumor cells (not observed in the matching normal tissue). For approximately half of the tumors, β-catenin data were available from our earlier studies[9], while for the rest, these results were generated in the present investigation.

EPS8 mutation analysis

All coding exons of the *EPS8* gene were examined by direct sequencing. The primer sequences and PCR conditions are given in Table 1. *EPS8* sequences were compared to that of GenBank accession number RefSeq NC_000012.10, and exon information was from Ensembl ENST00000389337. DNA mutation numbering is based on cDNA sequence where +1 corresponds to the A of the ATG translation initiation codon and the initiation codon is codon 1. Sequence changes reported here were present in sequence tracing from both the forward and reverse direction and were reproducibly found in 2 independent PCR products from cases of interest.

EPS8 methylation analysis

To search for CpG islands in the *EPS8* promoter, the EMBOSS CpG Plot program was used with default definitions (http://www.ebi.ac.uk/emboss). Two adjacent CpG islands were identified, together spanning 750 bp within and upstream of the untranslated exon number 1. This area was divided into two overlapping segments to screen cell lines and normal lymphocytes for methylation by bisulfite sequencing. The primers for the distal region were, forward, 5'-gggagatttttagggatttgatgg-3' and reverse, 5'-ccaaattatcaaaaccacaatcaaaatc-3', and for the proximal region (closest to and in part including the untranslated exon 1), forward, 5'-ttagttagttttgttagggtatttttgg-3' and reverse, 5'-ctaactactacctataaaatctaaaacc-3'. Only the distal region showed any evidence of methylation, which is why we focused on this region when designing methylation-specific PCR (MSP) assays for the studies of patient specimens.

MSP[11] was performed to separately amplify either methylated or unmethylated alleles from the distal region of the *EPS8* promoter (see above). Two alternative pairs of primers (MF1 + MR1, or MF3 + MR3) were used for the methylated reaction, and primers UF1 + UR1 for the unmethylated reaction. The primer sequences were: MF1, 5'-tggttattagatgcgttttgtttgtc-3', MR1, 5'-gtataaaaaacttcgcccccgacg-3'; MF3, 5'-ggtgttgtaatttgagcgttttttc-3', MR3, 5'-aacgtataaaaaacttcgccccg-3'; and UF1, 5'-ttggttattagatgtgttttgtttgtt-3', UR1 5'-ccaacaaaaataaacaccccaaaca-3'. DNA (1 μg) was modified with sodium bisulfite treatment (CpGenome DNA Modification kit, Chemicon) and subjected to MSP. MSP was performed in a volume of 25 μL containing 24 ng of bisulfite-modified template per reaction with HotStarTaq DNA polymerase (Qiagen). Cycling conditions were according to the manufacturers' standard cycling protocol for HotStarTaq DNA polymerase, with 35 cycles. Annealing temperatures were 58 ℃ for the methylated reaction MF1 + MR1, 64 ℃ for the methylated reaction MF3 + MR3, and 58 ℃ for the unmethylated reaction. MSP products were run through 2%-3% agarose gel, stained with ethidium bromide, and visualized with ultraviolet transillumination. All sodium bisulfite modifications and MSP runs were repeated at least twice. A negative control without template was included in each MSP run.

RESULTS

RKO is a special colon cancer cell line as it lacks constitu-

Table 1 Epidermal growth factor receptor pathway substrate 8 genomic primers (ENST00000389337)

Primer	Sequence	Primer for sequence	Tm	PCR fragment (bp)
EPS8ex1F	tcctggcagcaacacatatt	F	59	227
EPS8ex1R	ccaaatcaaattcccccaaa		62	
EPS8ex2F	aacccaacacaatgacctttttt		60	251
EPS8ex2R	tcactgcctcattccaaaca	R	60	
EPS8ex3F	gagatagccacatgataccaaca		59	195
EPS8ex3R	tgttcctcaagggtcactctaaa	F	60	
EPS8ex4F	tctttttccttttgccaat		56	280
EPS8ex4R	ttccatccattttcaaacaatc	R	58	
EPS8ex5F	gattgtttgaaaatggatggaa	F	59	261
EPS8ex5R	aaagctcccagacaatctgc		59	
EPS8ex6F	tcagacaaggaacaatcccttt	F	60	251
EPS8ex6R	tttttctaactctttggggaaaaa		60	
EPS8ex7F	agtaccacaagttgagttaattgat	F	55	264
EPS8ex7R	tcccaacccaaagtaagtgttc		60	
EPS8ex8F	ggcaaatggtcctccttttt	F	60	206
EPS8ex8R	ccagtgatctaaaggcgactc		59	
EPS8ex9F	tgggctgcttccttttctaa	F	60	280
EPS8ex9R	ctggagatctaaccaggcatt		58	
EPS8ex10F	cctctcctctcgcttatttca	F	58	234
EPS8ex10R	cacacccccacaaaatctat		57	
EPS8ex11F	gaccgtcccctctgtgtcta	F	60	229
EPS8ex11R	ccagacagacacttggggtta		60	
EPS8ex12F	cttgtttttgccatgggttt	F	60	265
EPS8ex12R	aaggcattataggtggtaaatgct		59	
EPS8ex13F	tatgccttcattccctcctg	F	60	297
EPS8ex13R	tgaataaaatgagaacttgcaatca		60	
EPS8ex14F	tgacctgagtgctgattcaaa	F	59	274
EPS8ex14R	gacactgtcacctctgttagcac		59	
EPS8ex15F	ctttaggaagagctagcagaat		54	250
EPS8ex15R	aatacttttgaaggaaagtttagttat	R	54	
EPS8ex16F	gggaacttctttcgtagaatgg	F	59	267
EPS8ex16R	aagagtgataacttcgtaaatgtgt		56	
EPS8ex17F	aaagtataatttgttttcctagcc	F	55	315
EPS8ex17R	tgcctccctgggaaacttac		61	
EPS8ex18F	ggggttctagagagggtgatgt	F	61	292
EPS8ex18R	tgttgtacacacagaattgcaaag		60	
EPS8ex19F	tttctcctttgtttgtaggcaat		59	256
EPS8ex19R	aatagttgtttccagagctttcaa	R	59	
EPS8ex20F	gcagcctgcacaagtcagta	F	60	203
EPS8ex20R	aatgccaaaaacaatggagtt		59	

EPS8: Epidermal growth factor receptor pathway substrate 8; PCR: Polymerase chain reaction; F: Forward; R: Reverse.

tive β-catenin regulated transcription compared to other colon cancer cell lines[8]. To detect genes that show the most remarkable differential expression in the RKO cell line compared to all the other cell lines (HCA7, KM12, LoVo, DLD1, HCT116, SW48, and LIM1215, each being mismatch repair-deficient like RKO), maximum deviance in signal between RKO and the remaining lines was calculated for each Affymetrix probe. When identifying putative over-expressed genes, the deviance was defined as the difference between the signal in RKO and the maximum signal in the other cell lines. In the case of under-expressed genes, the deviance was defined as the difference between the signal in RKO and the minimum signal in the other cell lines. The Affymetrix probe "202609_at" corresponding to the *EPS8* gene showed remarkable reduction in the RKO cell line when compared to the other cell lines. The signal detected in RKO was 118, whereas the other cell lines showed signals of 1454, 1361, 3792, 429, 683, 758 and 1804 (Log2 ratio-3.53).

Most patient samples were MSS apart from 5/43 (12%) which showed the MSI phenotype. By immunohistochemical analysis of clinical specimens, normal colonic mucosa showed dot-like supranuclear cytoplasmic expression pattern of EPS8 protein (Figure 1A). In some cases we noticed a gradient of expression with more intense staining at the luminal surface that faded away towards the intestinal crypts (Figure 1B). Colorectal adenomas and carcinomas showed three patterns of expression compared to their matching normal mucosae. 19 (4 adenoma, 15 carcinoma) out of 51 tumors (37%) showed tumor specific EPS8 protein loss, 5 (2 adenoma, and 3 carcinoma) out of 51 (10%) showed marked overexpression, while 27/51 tumors (53%) showed retained expression comparable to what was observed in the matching normal mucosae (Table 2, Figure 1C-F). However, there was no significant correlation between EPS8 expression pattern and β-catenin subcellular localization (in contrast to the finding in the RKO cell line), or tumor stage and location within the colon.

Table 2 Analysis of epidermal growth factor receptor pathway substrate 8 gene status and protein expression in uncultured tumor specimens *n* (%)

	Total number	Membranous β-catenin	LOH[1]	EPS8 protein loss	EPS8 mutation[2]	Methylation[3]
Sporadic carcinomas	22	10/22 (45)	7/19 (37)	11/22 (50)	0/11	0/7
Sporadic adenomas	15	4/10 (40)	6/15 (40)	4/14 (29)	0/4	ND
FCC-X	21	11/18 (61)	7/17 (41)	4/15 (27)	ND	ND
Total	58		20/51 (39)	19/51 (37)	0/15	0/7

[1]Based on informative (i.e., constitutionally heterozygous) cases; [2]Cases with protein loss by immunohistochemistry and/or presence of loss of heterozygosity (LOH) were selected for this analysis; [3]Cases were selected on the basis of immunohistochemical protein loss without LOH. ND: Not done; FCC-X: Familial colon cancer type X; EPS8: Epidermal growth factor receptor pathway substrate 8.

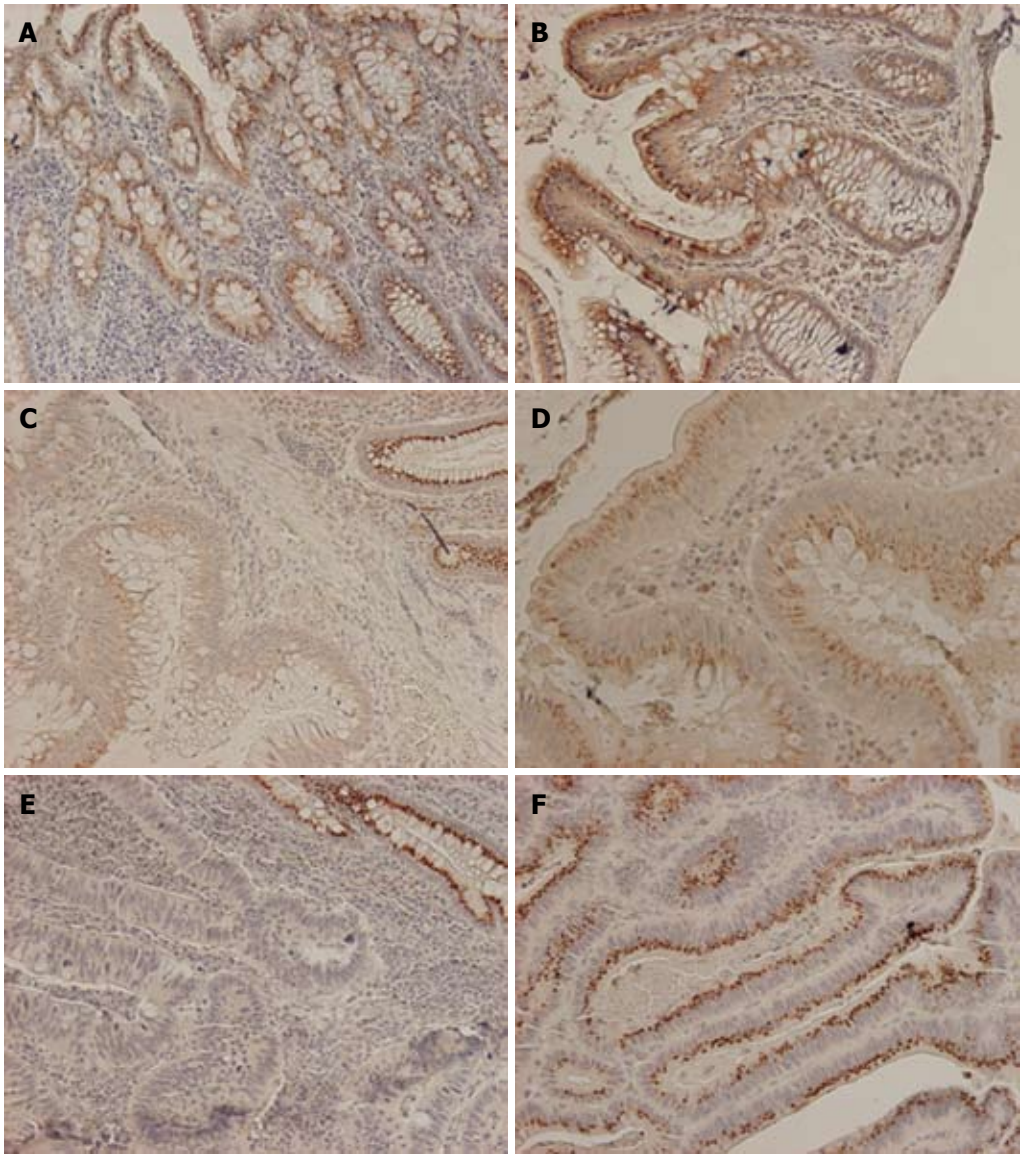

Figure 1 Epidermal growth factor receptor pathway substrate 8 immunohistochemistry. A: Normal colon mucosa with dot-like, supranuclear expression; B: Normal colon mucosa with clear gradient of expression stronger at the luminal aspect compared to the crypt bases; C: Marked reduction to complete loss of epidermal growth factor receptor pathway substrate 8 (EPS8) expression in adenoma (lower left) compared to normal mucosa (upper right); D: EPS8 positive adenoma; E: Marked reduction to complete loss of EPS8 expression in carcinoma (lower left) compared to normal mucosa (upper right); F: EPS8 positive carcinoma.

As possible mechanisms underlying expression changes, LOH, mutation, and promoter methylation were evaluated. We report LOH at EPS8 locus if, at least, one of the two markers D12S1580 and D12S1728 showed a clear cut LOH (40% or more reduction) while borderline-LOH (ratio reduction ranging from (25%-39%) at one marker only was ignored. Overall, 20/51 (39%) tumors showed *EPS8* locus LOH with similar frequencies in adenomas (40%)

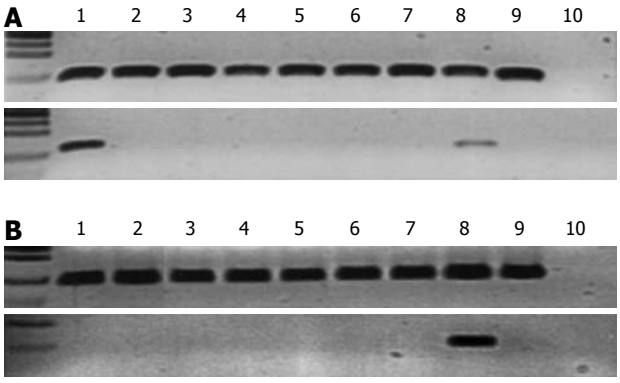

Figure 2 Methylation-specific polymerase chain reaction assays for epidermal growth factor receptor pathway substrate 8 gene. A: Cell lines analysis; upper panel is the unmethylated (UF1 + UR1) reaction and lower panel is the methylated (MF1 + MR1) reaction. Sample order in both panels from left to right is 1, RKO; 2, LoVo; 3, LIM1215; 4, HCA7; 5, HCT116; 6, KM12; 7, HCT15; 8, TK6 (a lymphoblastoid cell line); 9, unmethylated (negative) control; 10, water; B: Primary uncultured colon cancer analysis; upper panel is the unmethylated reaction and lower panel is the methylated reaction (MF3 + MR3). Sample order in both panels from left to right is 1-7, uncultured colon cancer specimens; 8, RKO (used as methylated control); 9, unmethylated (negative) control; 10, water.

and carcinomas (37%-41%) (Table 2). LOH was observed in 6/16 (38%) of informative tumors with absent EPS8 protein *vs* 9/39 (23%) of cases with retained or elevated protein expression ($P = 0.53$).

All coding exons and flanking intronic regions of the *EPS8* gene were examined by direct sequencing. The focus of this analysis was the cell line RKO and tumors with loss of EPS8 expression and/or LOH. We also included one of the control cell lines (HCA7 because of other special features[16]) We identified only one tumor specific missense mutation c.794C>T (p.R265C) in exon 8 in the RKO cell line. Since the matching normal tissue for the RKO cell line was not available, we further analyzed more than 100 normal DNA samples and none of them showed this change. To our knowledge, this nucleotide change is also not reported in any sequence or single nucleotide polymorphism (SNP) database. The nature of the amino acid change suggests that this is not likely to be a SNP since Arginine (R) is a positively charged, large polar amino acid that mostly prefers to substitute for the other positively charged amino acid Lysine, although in some circumstances it will also tolerate a change to other polar amino acids, but substitution with the small amino acid cysteine (C) is not tolerated in any cellular location[17]. We tested this particular substitution using the SIFT program (http://blocks.fhcrc.org/sift/SIFT.html) that sorts intolerant from tolerant amino acid substitutions based on evolutionary conservation, and cysteine substitution was regarded as intolerant.

Regarding *EPS8* methylation analysis, our very first observation was that the well-established human lymphoblastoid cell line TK6 was methylated in the distal region of the *EPS8* promoter, which indicated that the promoter was sensitive to methylation in general (Figure 2A). *EPS8* promoter methylation was examined by bisulfite sequencing in all cancer cell lines. These included cell lines in which the *MLH1* promoter was known to be methylated (RKO, KM12, HCA7) as well as cell lines with unmethylated MLH1 promoter (HCT15, HCT116, LoVo, LIM1215)[11]. Only RKO was methylated (Figure 2A). Encouraged by *EPS8* methylation in RKO, we designed MSP reactions to investigate *EPS8* methylation status in patient specimens of colorectal cancer. We focused on those cases that had no LOH at chromosome 12 markers (including cases that were uninformative for LOH), yet EPS8 protein was reduced or lost by immunohistochemistry, suggesting that there had to be alternative mechanisms for inactivation. There was no methylation in any of the seven tumor specimens analyzed, including five MSS tumors and two with MSI (Figure 2B). Given the lack of methylation in these samples of perhaps the highest interest, we did not extend these analyses to additional specimens.

DISCUSSION

Our data shed light on the role of *EPS8* in tumorigenesis in several important respects. EPS8 is involved in actin dynamics through its actin barbed-end capping activity and its ability to modulate Rac activity. Accordingly, EPS8 is crucial for the formation of actin networks that support cellular structures such as lamellipodia, filopodia, stress fibers and focal adhesions[18]. It appears that this is the most significant function of EPS8 in carcinogenesis also, since it did not colocalize with epidermal growth factor receptors but colocalized with F-actin in circular ruffles and at the leading edge of pancreatic cancer cells[19]. The data presented here support an important role of EPS8 in maturation and differentiation of the normal human colonic mucosa since normal colonic mucosa showed strong constitutive supranuclear cytoplasmic expression of EPS8 with increasing intensity towards the luminal surface away from the crypt base. These data are consistent with the well established role of EPS8 in the maturation of intestinal epithelium in *C. elegans*[5] and the previously described expression pattern in pancreatic ductal cells[19]. Potential roles of EPS8 in normal colonic epithelium might include the migration of proliferating cells from the bases of the crypt to the colonic luminal surface and/or stabilization of cell-cell junctions, as EPS8 was shown to be involved in cell-cell junction stability in fibroblasts[20], and EPS8 knockdown impaired actin cell-cell junction in confluent pancreatic cancer cells[19].

Regarding colon carcinoma, we noticed high levels of *EPS8* mRNA in all cell lines except RKO. However, immunohistochemical analysis of EPS8 protein in uncultured tumor biopsies showed that only around 10% of uncultured patient biopsies showed protein overexpression. This discrepancy between the cell line mRNA approach and patient biopsies' protein expression was consistent with the observation in pancreatic ductal adenocarcinomas[19] and may be explained by the apparent need of the cell lines to over-express motility and invasion markers. We are currently undertaking studies to ex-

plore the role of miRNA in posttranscriptional regulation of EPS8 protein expression. However, studies showed good correlation between mRNA and protein levels within the same tumor model[21].

The remarkable finding in this work was loss of EPS8 protein expression in subsets of colon adenoma and carcinoma. This finding is intriguing given the large number of published reports on EPS8 upregulation in different types of cancers, including those of the colon[7,19,22-24]. We also noted this upregulation at the levels of mRNA and protein expression in some tumors as discussed above. A careful analysis of the published reports shows, however, that most cases of EPS8 upregulation were characteristic of advanced stage and metastatic cancers[7,19,23,24]. The published literature suggests that EPS8 is most likely to be upregulated at the stage of metastasis. This hypothesis is best highlighted by the finding of EPS8 upregulation in the metastatic cell line SW620 as compared to its primary colon cancer cell line SW480[7]. These two cell lines are a well established model that have been used to study the markers associated with metastasis in colon carcinomas[25,26]. Similarly, the metastatic HN12 cells expressed high levels of EPS8 compared to its primary squamous cell carcinoma-derived cell line HN4[22]. In pancreatic cancer, cell lines from primary tumors had low levels of *EPS8* mRNA expression; cell lines from pancreatic cancer metastases had medium levels of *EPS8* mRNA expression; and a cell line derived from malignant ascites (AsPC-1) had high levels of *EPS8* mRNA expression[19]. These data could explain the apparent lack of mutation in our study, particularly in those tumors with loss of EPS8 protein which should leave a space for upregulation and overexpression at later stages; since reversion mutations are known to be extremely rare. In this regard, epigenetic and other regulatory mechanisms that could be easily reversed would be a preferable mode for controlling this gene expression status. Consistent with this, we noted the susceptibility of the *EPS8* promoter to methylation in the lymphoblastoid TK6 cells (Figure 2A) and its methylation in the RKO cell line associated with EPS8 mRNA underexpression. This is in agreement with RKO being a prototype of CpG island methylator phenotype that is usually observed in combination with MSI tumors[27]. We, however, did not observe promoter methylation in the primary tumors considered to have the highest a priori likelihood for methylation, suggesting that *EPS8* inactivation in these tumors occurred by other, as yet unknown mechanisms.

In conclusion, we report EPS8 loss of expression in colorectal adenomas and carcinomas and propose that EPS8 downregulation plays a role in the development of these tumors.

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COMMENTS

Background

Epidermal growth factor receptor pathway substrate 8 (EPS8) is a 97-kDa protein that is required for intestinal cell maturation. EPS8 was recently shown to be overexpressed in advanced stage human cancers including colon cancer cells. In this study, the authors analyzed EPS8 status in colorectal cancers.

Research frontiers

This work applies multiple approaches to gain insight into the expression status of EPS8 in colorectal cancer cell lines and primary tumors. Furthermore, it sheds light on the possible mechanisms of the observed expression alterations.

Innovations and breakthroughs

The remarkable finding in this work was loss of EPS8 protein expression in colorectal adenoma and carcinoma. This finding is intriguing given the previously published reports on EPS8 upregulation in different types of cancers, including those of the colon. Thus, the results show, for the first time, that EPS8 downregulation plays a role in the development of subsets of colorectal tumors.

Applications

The current findings could have applications in diagnosis and treatment of a subset of colon tumors. The observed expression differences of EPS8 here raise a note of caution about generalization of the previously reported findings of EPS8 overexpression in some tumors and re-emphasize the significance of personalized medicine in the treatment of cancer patients.

Peer review

It is an interesting study worth to be considered.

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BRIEF ARTICLE

Choice of approach for hepatectomy for hepatocellular carcinoma located in the caudate lobe: Isolated or combined lobectomy?

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Abstract

AIM: To investigate the significance of the surgical approaches in the prognosis of hepatocellular carcinoma (HCC) located in the caudate lobe with a multivariate regression analysis using a Cox proportional hazard model.

METHODS: Thirty-six patients with HCC underwent caudate lobectomy at a single tertiary referral center between January 1995 and June 2010. In this series, left-sided, right-sided and bilateral approaches were used. The outcomes of patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. The survival curves of the isolated and combined resection groups were generated by the Kaplan-Meier method and compared by a log-rank test.

RESULTS: Sixteen (44.4%) of 36 patients underwent isolated total or partial caudate lobectomy whereas 20 (55.6%) received a total or partial caudate lobectomy combined with an additional partial hepatectomy. The median diameter of the tumor was 6.7 cm (range, 2.1-15.8 cm). Patients who underwent an isolated caudate lobectomy had significantly longer operative time (240 min *vs* 170 min), longer length of hospital stay (18 d *vs* 13 d) and more blood loss (780 mL *vs* 270 mL) than patients who underwent a combined caudate lobectomy ($P < 0.05$). There were no perioperative deaths in both groups of patients. The complication rate was higher in the patients who underwent an isolated caudate lobectomy than in those who underwent combined caudate lobectomy (31.3% *vs* 10.0%, $P < 0.05$). The 1-, 3- and 5-year disease-free survival rates for the isolated caudate lobectomy and the combined caudate lobectomy groups were 54.5%, 6.5% and 0% and 85.8%, 37.6% and 0%, respectively ($P < 0.05$). The corresponding overall survival rates were 73.8%, 18.5% and 0% and 93.1%, 43.6% and 6.7% ($P < 0.05$).

CONCLUSION: The caudate lobectomy combined with an additional partial hepatectomy is preferred because this approach is technically less demanding and offers an adequate surgical margin.

Key words: Hepatocellular carcinoma; Hepatectomy; Caudate lobectomy; Caudate lobe; Combined resection

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INTRODUCTION

The caudate lobe is a segment of the liver that is surgically difficult to approach because of its deep location in the hepatic parenchyma[1-3]. The anatomic relationship of the caudate lobe to the hepatic vasculature was initially described by Couinaud. The caudate lobe is located anterior to the inferior vena cava (IVC), which may envelop this structure circumferentially. It extends to the hilum of the liver just posterior to the bifurcation of the portal vein. Cephalad, the caudate lobe lies posterior to the confluence of the left and middle veins as they enter the IVC on the left[4].

The caudate lobe is generally divided into three regions: the left Spiegelian lobe, the process portion and the paracaval portion. As these regions are supplied by different vasculobiliary branches of the portal triad and they are drained separately by branches of the hepatic veins, each region can be resected independently, thus making partial caudate lobectomy possible[5].

Caudate lobectomy is commonly indicated for hepatocellular carcinoma (HCC). It has been performed infrequently in the past, partly because of technical difficulties and the inadequate understanding of the anatomy[5-7]. Precise anatomic knowledge of the caudate lobe, and improvement in perioperative care and the surgical techniques have resulted in more performance of caudate lobectomies. However, caudate lobectomy remains a technical challenge, even for experienced hepatic surgeons. Caudate lobectomy is classified as total or partial lobectomy, and as isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy. This study aimed to evaluate the surgical outcomes of caudate lobectomy and the optimal surgical approach for HCC in the caudate lobe.

MATERIALS AND METHODS

Study subjects

Thirty-six patients with HCC underwent caudate lobectomy at the Department of Hepatobiliary Surgery and Liver Transplantation Surgery, Navy General Hospital between January 1995 and June 2010. Informed consent was obtained from each patient. Surgical outcomes for patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. The data were collected prospectively and analyzed retrospectively.

Procedure

The surgeries of this series were completed over the past 15 years. The choice of approach mainly depended on the prevailing conditions and surgeon's experience. Surgery was performed through a bilateral subcostal incision in eight patients, while a Mercedes-Benz incision was used in 28 patients. After an exploratory laparotomy, the liver was fully mobilized from all its peritoneal attachments. The liver was then assessed with intraoperative ultrasound. We carefully searched the abdominal cavity for the extent of local disease, extrahepatic metastases and peritoneal seedings. In this series, three approaches were used[8,9]: (1) a left-sided approach for tumors situated mainly in the Spiegelian lobe, or when a caudate lobectomy was combined with a left hepatectomy; (2) a right-sided approach for tumors situated mainly in the caudate process or paracaval portion, or when a caudate lobectomy was combined with a right hepatectomy; and (3) a bilateral approach for tumors situated in the whole caudate lobe. Although we started with one particular surgical approach in most patients, we had to combine different approaches to facilitate the caudate lobectomy. The suprahepatic and infrahepatic IVC was slung with vascular loops. Resection began with a pringle maneuver in cycles of 15/5 min of clamp/unclamp times. Total vascular exclusion was used only when patients had excessive bleeding from a lacerated IVC or hepatic vein. Liver resection was carried out by a clamp crushing method.

Statistical analysis

The survival curves of the isolated and combined resection groups were generated by the Kaplan-Meier method and compared with a log-rank test. To investigate the prognostic significance of the operative procedure, we performed a multivariate regression analysis with a Cox proportional hazard model, using a variable-selection method by a backward-elimination procedure. $P < 0.15$ was set as the cutoff for elimination. In the multivariate analysis, we chose 12 factors as potential confounders, considering their clinical significance and the results of previous reports[10,11]. Because any factors that are of potential importance can be incorporated into a multivariate analysis, whether or not they are statistically significant[12], we entered some nonsignificant factors in the univariate analysis into the model of the multivariate analysis in the present study. The 12 factors included: age (older *vs* younger than 65 years), sex, preoperative serum total bilirubin level (more *vs* less than 1 mg/dL), Child-Pugh class (A *vs* B), background liver status (cirrhosis *vs* noncirrhosis) as assessed histologically, tumor size (larger *vs* smaller than 30 mm), cancer spread (present or absent), tumor cell differentiation (well *vs* moderate or poor), serum-fetoprotein level (more *vs* less than 100 ng/mL), history of red blood cell transfusion (yes *vs* no), surgical margin (greater *vs* smaller than 5 mm) and tumor exposure (yes *vs* no). The Mann-Whitney *U* test and χ^2 test were used for the continuous and categorical data, respectively. All statistical analysis were performed using statistical software (SPSS 11.5 for Windows, SPSS, Inc., Chicago, IL). $P < 0.05$ was considered to be statistically significant.

Table 1 Patient characteristics

	Isolated caudate lobectomy group (n = 16)	Combined caudate lobectomy group (n = 20)	P value
Age (yr)	51 ± 14	48 ± 17	NS
Gender			NS
Male	12	15	
Female	4	5	
Liver cirrhosis			NS
Present	10	14	
Absent	6	6	
Child-Pugh class			NS
A	14	17	
B	2	3	
Liver function			
Albumin (g/dL)[1]	2.9	3.5	0.03
ALT (IU/L)[1]	54	32	0.04
Total bilirubin (mg/dL)[1]	1.2	0.8	0.04
Prothrombin time (%)[1]	75	79	NS
Location of the tumor			NS
Spiegel	2	3	
Paracaval portion	2	3	
Caudate process	1	2	
Spiegel + paracaval portion	3	4	
Paracaval portion + caudate process	3	2	
Complete caudate lobe	5	6	
Surgical margin (mm)			0.04
< 5 mm	5	1	
⩾ 5 mm	11	19	
α-Fetoprotein (ng/mL)[1]	23	25	
Cancer spread[2]			NS
Positive	3	5	
Negative	13	15	
Differentiation of tumor			NS
Edmondson Ⅰ	1	1	
Edmondson Ⅱ	5	4	
Edmondson Ⅲ	10	14	
Edmondson Ⅳ	0	1	
Tumor size, median (range), cm	6.1 (2.1-13.4)	7.5 (2.3-15.8)	NS

[1]Median; [2]Cancer spread was defined by presence of microscopic vascular invasion and/or intrahepatic metastasis. ALT: Alanine aminotransferase; NS: Not significant.

Table 2 Operative procedures

Operations	n (%)
Isolated caudate lobectomy	16 (44.4)
Complete caudate lobectomy	8
Partial caudate lobectomy	8
Concomitant procedures	
Partial IVC resection + repair	2
Approaches	
Left-side	2
Right-side	2
Bilateral	12
Combined caudate lobectomy	20 (55.6)
Complete caudate lobectomy + left hepatectomy	2
Complete caudate lobectomy + left lateral sectionectomy	2
Complete caudate lobectomy + right hepatectomy	1
Complete caudate lobectomy + right posterior hepatectomy	1
Partial caudate lobectomy + left hepatectomy	7
Partial caudate lobectomy + left lateral sectionectomy	3
Partial caudate lobectomy + right hepatectomy	2
Partial caudate lobectomy + right posterior hepatectomy	2
Concomitant procedures	
Partial IVC resection + repair	4
Approaches	
Left-side	4
Right-side	2
Bilateral	14

IVC: Inferior vena cava.

RESULTS

During the study period, 36 patients (28 males and 8 females) underwent caudate lobectomy for HCC. The median age was 49 years (range 31-74 years) , and 66.7% of the patients had liver cirrhosis. The median diameter of the tumor was 6.7 cm (range 2.1-15.8 cm). Tumors were present in all three parts of the caudate lobe in 11 patients, in the Spiegel lobe in five patients, in the paracaval portion in five patients, in the caudate process in three patients, in the paracaval portion and caudate process in five patients, and in the Spiegel and paracaval portion in 7 patients. The comparative data are shown in Table 1.

Surgical procedures

The operative procedures are listed in Table 2. Sixteen patients (44.4%) received an isolated complete or partial caudate lobectomy, whereas 20 (55.6%) underwent a complete or partial caudate lobectomy combined with an additional partial hepatectomy. Five patients required a partial resection and repair of the IVC because of tumor invasion into the anterior wall of the IVC. The left-sided, right-sided and bilateral approaches were used in 6, 4 and 26 patients, respectively.

Surgical outcomes

The surgical outcomes were compared between isolated caudate lobectomy and caudate lobectomy combined with an additional partial hepatectomy. The median operating time was 198 min (range 150-310 min) and the median blood loss was 620 mL (range 150-1470 mL). Patients that underwent an isolated caudate lobectomy had significantly longer operative time, length of hospital stay and blood loss than patients who underwent caudate lobectomy combined with an additional partial hepatectomy ($P < 0.05$). There were no perioperative deaths in both groups of patients. Patients that underwent an isolated caudate lobectomy had a higher complication rate than those who underwent caudate lobectomy combined with an additional partial hepatectomy (31.3% *vs* 10.1%, $P < 0.05$, Table 3).

The 1-, 3- and 5-year disease-free survival rates for the isolated caudate lobectomy and the combined caudate lobectomy groups were 54.5%, 6.5% and 0% and 85.8%, 37.6% and 0%, respectively ($P < 0.05$, Figure 1A). The corresponding overall survival rates were 73.8%, 18.5% and 0% and 93.1%, 43.6% and 6.7% ($P < 0.05$, Figure 1B). Multivariate analysis identified combined resection as significantly influencing the overall survival rate and the disease-free survival rate (Table 4).

Table 3 Surgical outcomes

	Isolated caudate lobectomy group (n = 16)	Combined caudate lobectomy group (n = 20)	P value
Time of vascular control, median (range), min	52 (32-68)	33 (25-39)	0.04
Blood loss, median (range), mL	780 (250-1470)	460 (150-980)	0.03
No. of patients with blood transfusion	14	11	0.02
Operative time, median (range), min	240 (170-310)	170 (150-225)	0.04
Hospital stay, median (range), d	18 (11-22)	13 (9-17)	0.04
Mortality	0	0	-
Complications			0.03
Liver failure	0	0	
Post-operative hemorrhage	2	0	
Bile leak	2	0	
Intra-abdominal collection	2	1	
Pleural effusion	1	1	

DISCUSSION

Although studies on caudate lobectomies have been increasingly reported, most of them were single case reports or small series reports[13-15]. Some series contained cases of caudate lobectomy carried out for microscopic involvement of hilar cholangiocarcinoma[16]. Comparative studies are very rare.

Caudate lobectomy is classified as a total or partial resection, and is also classified as an isolated or combined resection[17]. Several approaches have been described for caudate lobectomy, including the left-sided approach, right-sided approach, combined left- and right-sided approach and the anterior transhepatic approach. Peng *et al*[18] also described the retrograde approach for resecting tumors in the caudate lobe that had invaded the IVC. The selection of an appropriate surgical approach is essential for a safe caudate lobectomy. When the tumor is large or the IVC and/or major hepatic vein is compressed by the tumor, caudate lobectomy is technically very difficult and the resection has to be carried out using a combination of different approaches[19].

In an isolated caudate lobectomy, especially for a bulky tumor, it is important to recognize the danger of tearing the middle hepatic vein posteriorly when the caudate lobe is dissected away from this vein. To prevent major hemorrhage from a torn middle hepatic vein, the common trunk of the middle and left hepatic veins should be isolated and slung with a vascular loop before any attempt is made to dissect the caudate lobe within the tunnel formed by the IVC and the hepatic veins[20]. In a caudate lobectomy that is combined with either a right or left hepatectomy, a caudate lobectomy can be performed with little danger of bleeding from the middle hepatic vein since this vessel is usually controlled extrahepatically, or it can be sacrificed and resected together with the specimen. Thus, an isolated caudate lobectomy is technically more difficult than a caudate lobectomy combined with either a right or a left hepatectomy.

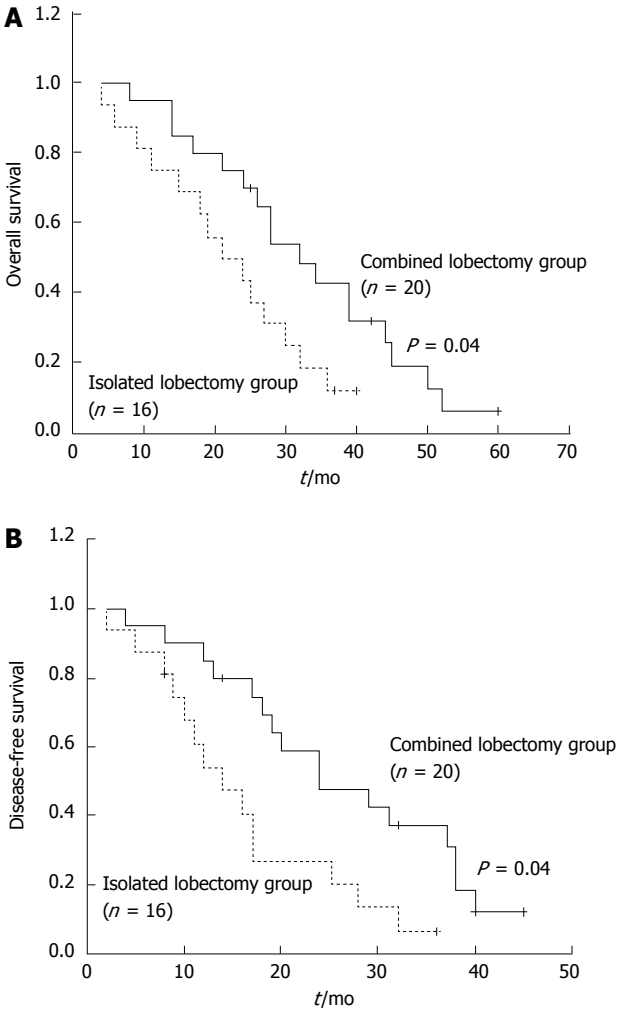

Figure 1 Overall survival rate curves and disease-free survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from caudate lobe. A: Overall survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from caudate lobe; B: Disease-free survival rate curves after isolated and combined resections for hepatocellular carcinoma originating from the caudate lobe.

The choice of isolated or combined resection is based primarily on the extent of HCC invasion and liver function reserve. The group that had an isolated resection of the tumor was characterized by well-differentiated, capsule intact, and poor liver function reserve, which could be easily removed. On the contrary, the group that had a combined resection of the tumor was characterized by poorly-differentiated, capsule incomplete, and better liver function reserve, which could be ablated with an extended resection to achieve the purpose of a complete resection.

We found that the isolated resection group had a worse long-term prognosis than the combined resection group. The main reasons were related to the following factors. First, the caudate lobe HCC was very close to the other lobe with limited growth space. Especially when the tumor was located in the paracaval part, it often infil-

Table 4 Multivariate analysis

Variables	Hazard ratio	95% CI	*P* value
Overall survival			
Absence of cancer spread[1]	0.44	0.24-0.69	0.007
Child-Pugh class A	0.86	0.66-1.33	0.17
Combined resection	0.57	0.32-0.92	0.04
Tumor size < 30 mm	0.61	0.32-1.05	0.08
Total bilirubin < 1 mg/dL	0.52	0.28-1.06	0.07
α-Fetoprotein < 100 ng/mL	0.61	0.33-1.19	0.07
Disease-free survival			
Absence of cancer spread[1]	0.61	0.37-0.82	0.001
Combined resection	0.66	0.42-0.94	0.03
Negative tumor exposure	0.39	0.20-0.77	0.04
Total bilirubin < 1 mg/dL	0.56	0.37-0.89	0.02

[1]Cancer spread was defined by presence of microscopic vascular invasion and/or intrahepatic metastasis.

trated the other lobe, such as segment Ⅳ, Ⅴ, Ⅵ, Ⅶ or Ⅷ. Due to the unclear boundary, an isolated resection of the tumor could not achieve a complete resection. Second, the caudate HCC was often close to the main branch of the main portal and hepatic veins, which increased the likelihood of vascular invasion leading to an inadequate surgical margin. A caudate lobe resection combined with the other lobe could obtain a clear exposure and acquire a more adequate surgical margin. Third, from the no-touch point of view, repeated over-turning and pulling on the caudate lobe can cause HCC cells to transfer to other locations along the portal vein and hepatic vein, increasing the possibility of metastasis in the isolated resection. Although the anterior approach can avoid this problem, its application is limited by varying degrees of liver cirrhosis. The anterior approach required segment Ⅳ resection, which prolonged the operation time and increased the amount of bleeding[21]. Obtaining a negative margin may not be easy particularly in large and very large HCC, especially for those located in the caudate lobe[22-24]. Therefore, the style of the combined resection can solve the above problem, which is an optimal method. We advocate that the caudate lobe should be ablated from the combined adjacent lobe to get an adequate margin and reduce the stretching and compression of the tumor, thereby achieving a good long-term prognosis.

If confounders in a multivariate analysis are limited only to the significant factors in a univariate analysis, some factors, which are not significant despite their potential importance, may be excluded. Therefore, according to Tralhão *et al*[25], we chose 12 factors as confounders, after weighing their clinical importance, whether or not they were significant in the univariate analysis. Indeed, this method was also adopted in a previous study[26,27]. The present study indicated that anatomic resection would be a suitable option of choice for HCC. Our multivariate analysis showed that liver function was an important prognostic factor for the overall survival, though the Child-Pugh class between the two groups showed no difference. In the other study, we found that segmentectomy or lobectomy might be recommended as an initial treatment for patients with good hepatic function and a solitary hepatic nodule because such patients have a chance of achieving long-term survival and wider surgical resections could minimize the chance of microscopic residual tumors or occult metastases[28-32].

Approaches to a caudate lobectomy thus depend largely on the size and location of the lesion and liver functional reserve. For patients with sufficient liver functional reserve, partial or complete caudate lobectomy combined with other partial hepatic resections is preferred because such an operation is technically less demanding. For patients with a poor liver function, we are left with no choice but to carry out an isolated caudate lobectomy. HCC originating from the caudate lobe is relatively rare. As the study sample is small, a more accurate conclusion requires a multi-center randomized controlled study to confirm our results.

COMMENTS

Background

The caudate lobe is a segment of the liver that is surgically difficult to approach because of its location deep in the hepatic parenchyma, which is surrounded by branches of the porta hepatis, the hepatic veins and the inferior vena cava (IVC). Caudate lobectomy is commonly indicated for hepatocellular carcinoma (HCC). Currently, caudate lobectomy remains a technical challenge, even for experienced hepatic surgeons. This study gives some instructions for hepatectomy for HCC located in the caudate lobe, with the choice of isolated or combined lobectomy.

Research frontiers

Caudate lobectomy is classified as total or partial lobectomy; it is also classified as isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy. The selection of an appropriate surgical approach is essential for a safe caudate lobectomy.

Innovations and breakthroughs

Although increasing numbers of studies on caudate lobectomy have been reported in the medical literature, most are single case reports or small series studies. Some series contained cases of caudate lobectomy carried out for microscopic involvement of hilar cholangiocarcinoma. Comparative studies are very rare. In this paper, 36 patients with HCC underwent caudate lobectomy at a single tertiary referral center between January 1995 and June 2010. The surgical outcomes of patients who underwent isolated caudate lobectomy or caudate lobectomy combined with an additional partial hepatectomy were compared. For patients with sufficient liver functional reserve, caudate lobectomy combined with an additional partial hepatectomy is preferred because such an approach is technically less demanding and offers an adequate surgical margin. For patients with a marginal liver functional reserve, the viable surgical option is an isolated caudate lobectomy.

Applications

This study showed that, in patients with sufficient liver functional reserve, a caudate lobectomy combined with an additional partial hepatectomy is preferred because such an approach is technically less demanding and achieves adequate surgical margin. However, for patients with marginal liver functional reserve, the viable surgical option is an isolated caudate lobectomy.

Terminology

Caudate lobectomy is classified as total or partial resection, and is also classified as an isolated or combined resection. Several approaches have been described for caudate lobectomy, such as the left-sided approach, right-sided approach, combined left- and right-sided approach and the anterior transhepatic approach. Recently, a retrograde approach for resecting tumors in the caudate lobe that have invaded the IVC has also been described.

Peer review

This manuscript emphasizes the optimal surgical approach for HCC in the caudate lobe. The manuscript sections are very clearly described and the conclusion is an opened door for further investigation. They observed that the 16 patients who underwent isolated lobectomy had longer operative times, greater blood loss, a higher complication rate, longer hospital stays and higher mortality. They concluded that, in patients with adequate functional reserve, combined hepatectomy is the preferred choice.

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BRIEF ARTICLE

Normal carcinoembryonic antigen indicates benefit from perioperative chemotherapy to gastric carcinoma patients

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Abstract

AIM: To evaluate pretreatment serum carcinoembryonic antigen (CEA) as a predictor of survival for patients with locally advanced gastric cancer receiving perioperative chemotherapy.

METHODS: We retrospectively studied a cohort of 228 gastric cancer patients who underwent D2 gastrectomy combined with chemotherapy at the Sun Yat-sen University Cancer Center between January 2005 and December 2009. Among them, 168 patients received 6-12 cycles of oxaliplatin-based adjuvant (post-operative) chemotherapy, while 60 received perioperative chemotherapy (2 cycles of FOLFOX6 or XELOX before surgery and 4-10 cycles after surgery). Serum CEA was measured using an enzyme immunoassay. The follow-up lasted until December 2010.

RESULTS: In the group that had elevated serum CEA, the difference in survival time between patients receiving perioperative chemotherapy and those receiving adjuvant chemotherapy had no statistical significance (P > 0.05). However, in the group that had normal serum CEA, patients receiving perioperative chemotherapy had a longer survival time. In multivariate analysis, T staging and lymph node metastatic rate were independent prognostic factors for the patients. Perioperative chemotherapy improved the overall survival of patients who had a normal pretreatment CEA level (P = 0.070).

CONCLUSION: Normal pretreatment serum CEA is a predictor of survival for patients receiving perioperative chemotherapy.

Key words: Carcinoembryonic antigen; Perioperative chemotherapy; Prognosis; Gastric adenocarcinoma; Survival

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INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. It is the second leading cause of cancer deaths in the world[1-3], and most of those patients are diagnosed at an advanced stage of disease[4,5]. Surgery is the main

treatment for gastric cancer. Many meta-analyses have demonstrated that adjuvant (post-operative) chemotherapy can improve the prognosis for gastric cancer patients[6-8], and in some prospective clinical trials, adjuvant chemotherapy has improved the prognosis of patients with locally advanced gastric cancer[9-11]. The Cunningham trial showed for the first time that perioperative chemotherapy (treatment both before and after surgery) is superior to surgery alone in treating gastric cancers. Further studies showed that preoperative chemotherapy combined with chemoradiotherapy provided substantial responses that improved the prognosis[12-14].

Carcinoembryonic antigen (CEA) was first identified in 1965 by Gold and Freedman in human colon cancer tissue extracts[15]. In the last two decades, CEA has been widely used as a tumor marker in the diagnosis and monitoring of some malignancies[16]. Since the 1990s, tumor markers including CEA, carbohydrate antigen 19-9, and others have been widely used to monitor gastric cancer progression and even to assess the prognosis of gastric cancer patients, although their specificities have not been satisfactory[17-20]. The controversial conclusions resulting from the use of these biomarkers are therefore understandable[21]. In the present study, we retrospectively evaluated the predictive value of pretreatment serum CEA in patients with late-stage gastric cancer in China.

MATERIALS AND METHODS

Patient inclusion and exclusion criteria

Inclusion criteria: (1) age: 20 to 75 years; World Health Organization performance status 0 to 1; (2) histologically proven adenocarcinoma of the stomach; T3 or T4 tumor based on endoscopic ultrasound; no evidence of distant metastases or of disease considered nonresectable by endoscopic ultrasonography, computed tomography (CT), or extended diagnostic laparoscopy; (3) no prior gastric surgery; (4) no previous radiotherapy or other treatments, including immunotherapy or Chinese traditional medicine; (5) no uncontrolled infectious or cardiac disease; adequate hepatic and renal functions; and (6) no synchronous or metachronous cancers.

Exclusion criteria: (1) age: older than 75 years or younger than 20 years; (2) hepatic, renal, pulmonary, or cardiac dysfunction; and (3) severe postoperative complications, such as anastomosis leakage or anastomosis stenosis, that may cause malnutrition or make the patients intolerant to postoperative chemotherapy.

Patient characteristics

We included 228 patients who underwent D2 gastrectomy at the Sun Yat-sen University Cancer Center between January 2005 and December 2009. Among them, 173 patients had a normal pretreatment serum CEA (≤ 5 ng/mL) and 55 patients had elevated pretreatment serum CEA (> 5 ng/mL). Sixty patients among both CEA groups (43 with normal serum CEA and 17 with elevated serum CEA) received oxaliplatin-based perioperative chemotherapy, with 2 cycles before surgery and 4-10 cycles of the same regimen after surgery. The exception was 6 patients suffering from progressive disease who received second-line chemotherapy for 4-6 cycles (see Treatment section). Among both CEA groups, 168 patients received only adjuvant chemotherapy (Figure 1). The clinicopathological characteristics of all patients are presented in Table 1.

Table 1 Clinicopathologic characterization of patients with gastric adenocarcinoma treated by surgery in combination with chemotherapy

Data	Normal serum CEA (median 52 yr, range: 22-74 yr) *n* (%)	Elevated serum CEA (median 54 yr, range: 32-73 yr) *n* (%)	*P*
Sex			0.090
Male	116 (67.1)	44 (80.0)	
Female	57 (32.9)	11 (20.0)	
Tumor location			0.115
Upper	52 (30.0)	26 (47.3)	
Middle	42 (24.3)	12 (21.8)	
Lower	68 (39.3)	15 (27.3)	
Total	11 (6.4)	2 (3.6)	
Histological grade			0.128
G1	1 (0.6)	0 (0)	
G2	31 (17.9)	18 (32.7)	
G3	112 (64.7)	29 (52.7)	
G4	29 (16.8)	8 (14.6)	
Tumor size			0.053
≤ 2 cm	23 (13.3)	1 (1.8)	
2 cm < diameter ≤ 5 cm	98 (56.6)	36 (65.5)	
> 5 cm	52 (30.1)	18 (32.7)	
Boarrman type			0.093
Ⅰ	3 (1.7)	0 (0)	
Ⅱ	90 (52.0)	20 (36.4)	
Ⅲ	69 (39.9)	28 (50.9)	
Ⅳ	11 (6.4)	7 (12.7)	
Pathological T staging[1]			0.664
T0	6 (3.5)	1 (1.8)	
T3	156 (90.2)	49 (89.1)	
T4	11 (6.4)	5 (9.1)	
Lymph node metastasis rate			0.951
0	12 (6.9)	3 (5.5)	
0 < r ≤ 0.1	29 (16.8)	8 (14.5)	
0.1 < r ≤ 0.3	50 (28.9)	17 (30.9)	
r > 0.3	82 (47.4)	27 (49.1)	
Surgery			0.313
Radical	165 (95.4)	50 (90.9)	
Palliative	8 (4.6)	5 (9.1)	
Chemotherapy			0.384
Adjuvant	130 (75.1)	38 (69.1)	
Perioperative	43 (24.9)	17 (30.9)	

[1]Pathological T staging was based on the 6th Union for International Cancer Control's staging systems for gastric cancer. CEA: Carcinoembryonic antigen.

Treatment

The two cycles of preoperative chemotherapy included the XELOX and FOLFOX regimes. All the chemotherapy regimens were used under standard protocols. The XELOX regimen consisted of oxaliplatin at 130 mg/m^2 (i.v. drip, day 1) and capecitabine at 1000 mg/m^2 (oral,

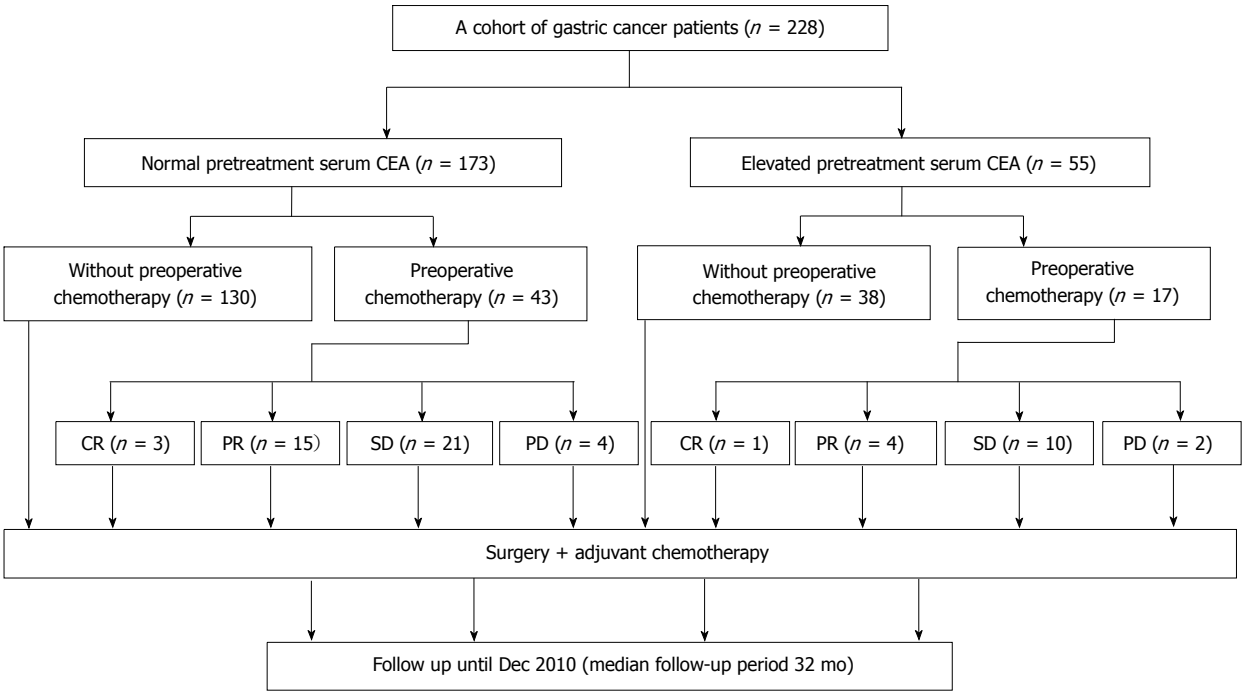

Figure 1 The treatment subgroups of the cohort of 228 patients in this retrospective study. CEA: Carcinoembryonic antigen; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

day 1-14), followed by one week of no treatment. Starting on day 22, the cycle was repeated, and surgery took place between day 43 and 47.

The FOLFOX6 regimen started on day 1 with oxaliplatin at 100 mg/m^2 (i.v. drip) with folic acid at 400 mg/m^2 (racemic) or 200 mg/m^2 (L-form), plus 5-fluorouracil (5-FU) as a 400 mg/m^2 bolus, followed by 2400 mg/m^2 of 5-FU as a continuous 46 h infusion. When the infusion was completed, there was no further treatment through day 14. On day 15, the cycle was repeated, with surgery taking place between day 30 and day 33.

After surgery, all of the patients received adjuvant chemotherapy starting within 2-4 wk. The median number of cycles for each regimen was 9 for FOLFOX6 (range: 7-12) and 7 for XELOX (range: 6-8). The six progressive disease patients received paclitaxel plus 5-FU (1 patient), docetaxel plus 5-FU (2 patients), or S-1 oral administration (3 patients) chemotherapy; these patients received a median of 5 cycles (range: 4-6).

Chemotherapy response evaluation

Assessment of the response to preoperative chemotherapy was based on the reduction of primary tumor size (as measured by endoscopic ultrasonography and CT scan) and the Response Evaluation Criteria in Solid Tumors criteria.

Complete response: Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have a reduction of the short axis to less than 10 mm.

Partial response: At least a 30% decrease in the sum of diameters of target lesions, taking as a reference the baseline sum of diameters.

Stable disease: Neither sufficient shrinkage to qualify as a partial response nor sufficient increase to qualify as progressive disease.

Progressive disease: At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum in the study (which may include the baseline sum). The sum must also show an absolute increase of at least 5 mm.

Patient follow-up

After treatment, the patients were monitored every 3 mo for the first 2 years, then every 6 mo thereafter. Telephone calls and letters were used to assess patients who could not be physically present. Complete data were collected from all 228 patients until December 2010. The follow-up period ranged from 8 mo to 59 mo (median, 32 mo). The total follow-up times are shown in Figure 2A.

Statistical analysis

The χ^2 test was used to compare categorical variables between the normal and elevated serum CEA groups. Univariate survival analysis was performed using the Kaplan-Meier method. Survival curves were compared with the log-rank test. Multivariate statistical survival analysis was performed using Cox regression. Analysis were performed with SPSS software version 16.0 for Windows

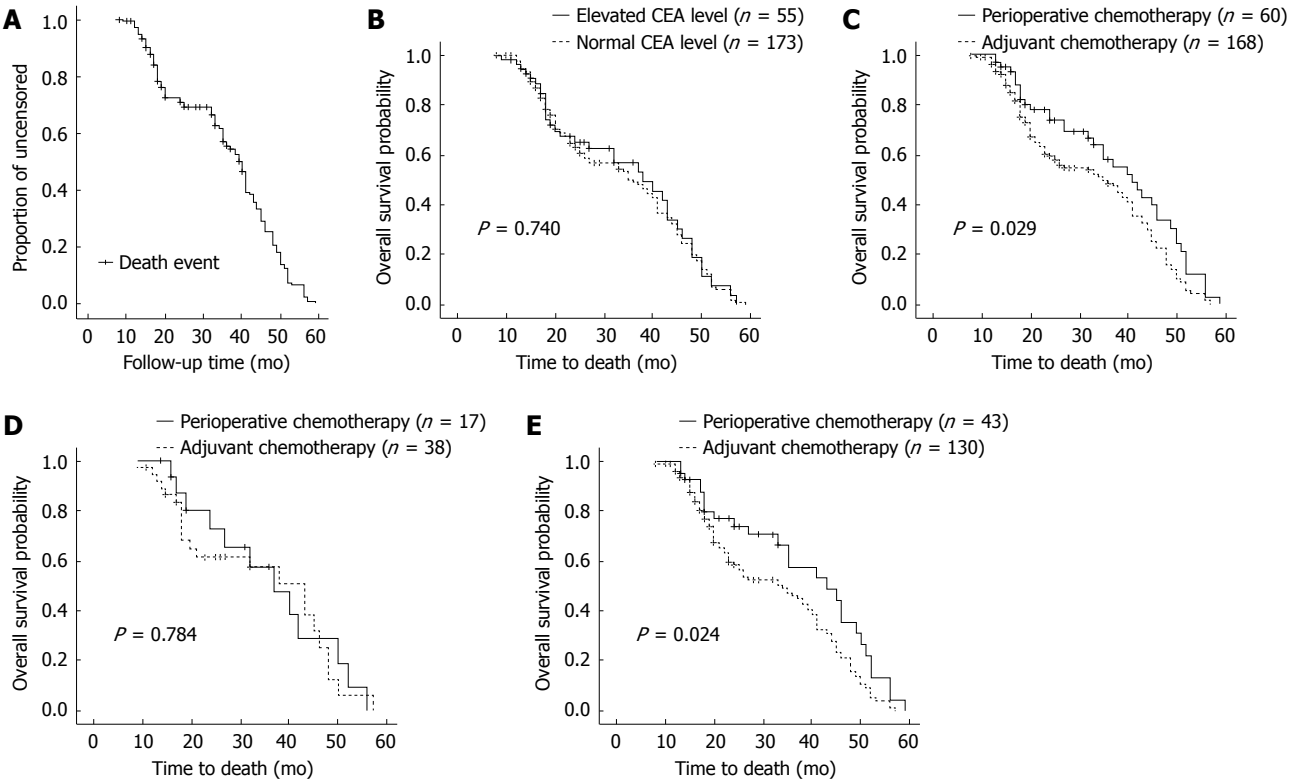

Figure 2 Survival curves for carcinoembryonic antigen patients with different treatments. A: The 228 patients in follow-up over this study; follow-up times ranged from 8 to 59 mo (median, 32 mo); B: Survival curves for the elevated carcinoembryonic antigen (CEA) patients and the normal CEA patients, with two-year survival rates of 57% and 51%, respectively, and no statistically significant difference between the groups (P = 0.740); C: Survival curves for patients receiving perioperative chemotherapy or adjuvant chemotherapy, with two-year survival rates of 58% and 50%, respectively. The difference is statistically significant (P = 0.029); D: Survival curves for the elevated CEA patients who received perioperative or adjuvant chemotherapy, with two-year survival rates of 58% and 58%, respectively, with no statistical significance (P = 0.784); E: Survival curves for normal CEA patients who received perioperative or adjuvant chemotherapy, with a two-year survival rate of 59% and 48%, respectively. The difference is statistically significant (P = 0.024).

(SPSS, Inc., Chicago, IL, United States). Statistical significance was defined as $P < 0.05$.

RESULTS

There was no statistically significant difference in overall survival between the normal CEA group (n = 173) and the elevated CEA group (n = 55). The survival curves are shown in Figure 2B.

The efficacy of preoperative chemotherapy was evaluated. Among these 60 patients, 4 (6.67%) had complete clinical response and 19 (31.7%) had partial clinical response, yielding an overall response rate of 40.0%. The response rates of the patients who had normal pretreatment serum CEA *vs* those who had elevated CEA were not significantly different, although patients with normal CEA had a higher response rate (complete response + partial response) (41.9% *vs* 29.4%). These results are shown in Table 2.

The 60 patients who received perioperative chemotherapy (i.e., preoperative plus adjuvant) had a significantly better overall survival rate than the 168 who received only adjuvant chemotherapy, with median survival time of 41 mo for the perioperative group *vs* 35 mo for the adjuvant group (P = 0.029). The survival curves are shown in Figure 2C. For the patients with elevated serum CEA, there was no significant difference in overall survival rate between perioperative chemotherapy (n = 17) and adjuvant chemotherapy (n = 38, P = 0.784, Figure 2D). For the patients with normal serum CEA, the overall survival rate was significantly better in the perioperative group (n = 43) and the median survival time was 43 mo *vs* 34 mo for the adjuvant group (n = 130, P = 0.024). The survival curves are shown in Figure 2E.

In univariate analyses, perioperative chemotherapy, T staging, and the lymph node metastasis rate significantly correlated with overall survival (Table 3). In multivariate analysis, T staging and the lymph node metastatic rate were independent prognostic factors. Perioperative chemotherapy improved the overall survival of patients who had normal pretreatment serum CEA (Table 4).

DISCUSSION

Perioperative chemotherapy, although it is a large physical and psychological burden, has been proven to be effective for some gastric cancer patients. The European Organization for Research and Treatment of Cancer Randomized Trial 40 954 showed no survival benefit from preoperative chemotherapy compared with surgery alone for locally advanced cancer[22]. However, this study had low statistical power; a high number of proximal

Table 2 The efficacy of preoperative chemotherapy on locally advanced gastric cancer patients *n* (%)

Pretreatment serum CEA	Response to preoperative chemotherapy			
	Complete response	Partial response	Stable disease	Progressive disease
Normal	3 (7.0)	15 (34.9)	21 (48.8)	4 (9.3)
Elevated	1 (5.9)	4 (23.5)	10 (58.8)	2 (11.8)

CEA: Carcinoembryonic antigen.

Table 3 Univariate analysis of overall survival in all patients and in patients with normal pretreatment serum carcinoembryonic antigen

Variable	No. of patients	2-yr survival rate (%)	Median survival (mo)	*P* value
All patients				
Pathological T staging				0.001
T0	7	86	52	
T3	205	52	37	
T4	16	36	27	
Lymph node metastasis				0.001
0	15	79	48	
0 < r ≤ 0.1	37	68	31	
0.1 < r ≤ 0.3	67	42	32	
r > 0.3	109	50	33	
Chemotherapy				0.029
Adjuvant	168	50	35	
Perioperative	60	58	41	
Normal-CEA patients				
Pathological T staging				0.001
T0	6	83	50	
T3	156	50	35	
T4	11	39	17	
Lymph node metastasis				0.002
0	12	74	44	
0 < r ≤ 0.1	29	70	45	
0.1 < r ≤ 0.3	50	38	26	
r > 0.3	82	49	33	
Chemotherapy				0.024
Adjuvant	130	48	34	
Perioperative	43	59	43	

CEA: Carcinoembryonic antigen.

Table 4 Multivariate analyses (Cox regression model) of overall survival of all patients and of patients having a normal pretreatment serum carcinoembryonic antigen

Variable	Hazard ratio	95% CI	*P* value
All patients			
Perioperative chemotherapy	0.723	0.501–1.044	0.084
T staging	1.422	1.067–1.896	0.016
Lymph node metastasis rate	1.302	1.101–1.539	0.002
Normal-CEA patients			
Perioperative chemotherapy	0.670	0.434–1.033	0.070
T staging	1.443	1.041–2.000	0.028
Lymph node metastasis rate	1.274	1.053–1.542	0.013

CEA: Carcinoembryonic antigen.

gastric cancers (which involved the gastroesophageal junction and were different from most of the cases in endemic areas); and an increased R0 resection rate, indicating a better outcome in those patients suffering from early-stage gastric cancer. In China, most gastric cancer patients are diagnosed as having locally advanced disease, suggesting that different treatments should be considered for increasing the survival of the patients. However, there is no reliable marker to determine which patients with advanced gastric cancer can benefit from perioperative chemotherapy. The goal of the present study was to determine whether the pretreatment serum CEA level could be used as a marker to select patients for this aggressive treatment.

Our study revealed that perioperative chemotherapy can improve overall survival in patients with advanced gastric cancer. Dividing the patients in two groups based on their pretreatment serum CEA, we found that perioperative chemotherapy improved the survival rate only for patients with a normal level of pretreatment serum CEA.

Although the biological functions of CEA are not fully known, the close correlation of CEA with cancer aggressiveness has been known for decades. Higher preoperative CEA correlates with more aggressive gastric cancer and a lower patient survival rate[23]. Our findings imply that patients with elevated CEA might have gastric cancers more resistant to chemotherapy, resulting in no survival benefit even from aggressive chemotherapy. CEA has been reported to have roles in homotypic adhesion and cellular aggregation[24], and it cooperates with Myc and Bcl-2 in cellular transformation[25]. In colon cancer, CEA is up-regulated in the microadenoma stage in the colon of patients with APC mutations[26], and CEA plays antiapoptotic and prometastatic roles in colon cancer cells[27]. Overexpression of CEA can protect tumor cells from apoptosis induced by loss of cell contact with the extracellular matrix (anoikis)[28].

Interestingly, those gastric cancer cells expressing alpha-fetoprotein (which is another oncofetal antigen) show P-glycoprotein overexpression and drug resistance in both animal models and human cancer[29,30]. In some case reports, drug-resistant patients always had elevated serum CEA[31,32], and CEA overexpression was observed in multidrug-resistant breast carcinoma cell lines[33]. The CEA promoter (AdCEAIacZ) can increase the IC50 of ganciclovir against gastric cancer cell lines by improving CEA production[34]. All of these pieces of evidence suggest that CEA may induce or promote drug resistance in cancer cells.

This study is retrospective, with its own weaknesses such as confounding factors and low persuasiveness. The cycles of adjuvant chemotherapy were different among the patients, and there were two main chemotherapy regimens: XELOX and FOLFOX6. We believe that the study would be more convincing if there had been a standard regimen, although most investigators report the efficacy of these two regimens as having no statistically significant difference in gastric cancer patients. Thus, more randomized controlled trials are needed to confirm

that pretreatment serum CEA can be used as a marker to select patients for the aggressive perioperative treatment or to find other markers for assigning patients to an appropriate treatment.

To our knowledge, there is no strong evidence that CEA is a marker of drug resistance in gastric cancer. More experiments and clinical trials are needed to validate whether CEA levels can predict such drug resistance. In summary, our study showed that only patients with normal pretreatment serum CEA obtained a survival benefit from cytotoxic perioperative chemotherapy. The role of CEA in the drug resistance of gastric cancers warrants further exploration.

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COMMENTS

Background

Perioperative chemotherapy has been proven to be effective for some gastric cancer patients. However, there is no reliable marker for determining which patients with advanced gastric cancer can benefit from perioperative chemotherapy.

Research frontiers

In the last two decades, carcinoembryonic antigen (CEA) has been widely used as a tumor marker in the diagnosis and monitoring of some malignancies. The research hotspot is to determine whether pretreatment serum CEA can be used as a marker to select patients for this aggressive perioperative treatment.

Innovations and breakthroughs

The study revealed that perioperative chemotherapy can improve overall survival in patients with advanced gastric cancer. After dividing the patients in two groups based on their pretreatment serum CEA, the authors found that perioperative chemotherapy improved the survival rate only for patients with a normal level of pretreatment serum CEA.

Applications

The study results suggest that normal pretreatment serum CEA is a predictor of survival benefit for the patients receiving perioperative chemotherapy.

Peer review

This is an interesting paper aimed to evaluate the role of pretreatment serum CEA level as a predictor of survival for patients with locally advanced gastric cancer receiving neoadjuvant chemotherapy. This retrospective study is well conducted and well written.

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CASE REPORT

Thrombosis of celiacomesenteric trunk: Report of a case

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Abstract

Here we present the case of a 79-year-old woman who complained of acute abdominal pain, vomiting and diarrhoea. Laboratory exams demonstrated a severe metabolic imbalance. Abdominal X-rays showed bowel overdistension and pneumatosis of the stomach wall. Abdominal tomography revealed infarction of the stomach, duodenum and small bowel due to thrombosis of the celiacomesenteric trunk. Exploratory laparotomy revealed ischemia of the liver, spleen infarction and necrosis of the gastro-intestinal tube (from the stomach up to the first third of the transverse colon). No further surgical procedures were performed. The patient died the following day. To our knowledge, this is the first reported case about severe gastro-intestinal ischemia due to thrombosis of the celiacomesenteric trunk, a rare anatomic variation of the gastrointestinal vascularisation.

Key words: Celiacomesenteric trunk; Celiac trunk; Thrombosis; Anomalies; Gastrointestinal vascularisation

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INTRODUCTION

The majority of the blood supply of the gastrointestinal tract is provided by the anterior branches of the abdominal aorta: celiac trunk and superior mesenteric artery. Usually, the aforementioned branches arise independently from the abdominal aorta, the first one at the level of the twelfth thoracic vertebra, the second one at the level of the first lumbar vertebra. Anomalies of vascularisation of the gastro-intestinal tract are frequent, but the presence of the celiacomesenteric trunk (derived by common origin of celiac trunk and superior mesenteric artery) is rare. The injury of the trunk can have lethal effects on the organism. Here we describe the first reported case of severe ischemia of the gastrointestinal tract due to thrombosis of the celiacomesenteric trunk.

CASE REPORT

In September 2011, a 79-year-old woman arrived at the emergency department of San Biagio hospital with severe and diffuse abdominal pain and tenderness, more marked in the lower abdominal quadrants, with signs of peritoneal irritation. The area of hepatic dullness was

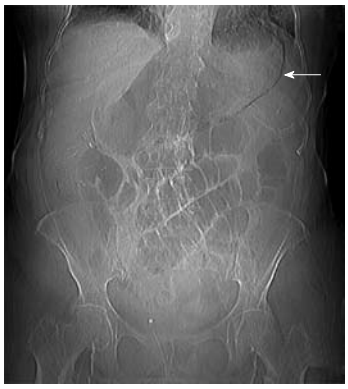

Figure 1 Abdominal X-rays. Gaseous overdistension of the small bowel. The arrow points the pneumatosis of the stomach wall.

present at percussion and intestinal peristalsis was diminished. The patient was apyretic and eupnoic at rest. Laboratory analyses demonstrated increased inflammatory markers (neutrophilic leukocytes, polymerase chain reaction), acute renal failure (creatinine: 5.85 mg/dL) and severe metabolic acidosis. No pathological signs were found at chest X-rays, whereas abdominal X-rays revealed gaseous overdistension of the small bowel and pneumatosis of the stomach wall (Figure 1).

The radiological findings were subsequently confirmed by computed tomography of the abdomen performed without iodinated contrast due to renal failure of the patient. Tomography demonstrated pneumatosis of the wall of the stomach, duodenum and small bowel. Air was also present within the superior mesenteric and portal veins with intraparenchymal distribution to ventral portions of the left liver and fourth segment (Figure 2A), because of the advanced stage of arterial infarction. The axial scan evidenced the thrombosis of the celiacomesenteric trunk, which justified the radiological findings (Figure 2B); no further arterial vessel directed to the gastro-intestinal tract was identified by tomography.

Exploratory laparotomy showed ischemia of the liver, spleen infarction and necrosis of the stomach, duodenum, small bowel and large intestine (from the caecum to the first third of transverse colon). No further surgical procedures were performed. The patient died the following day.

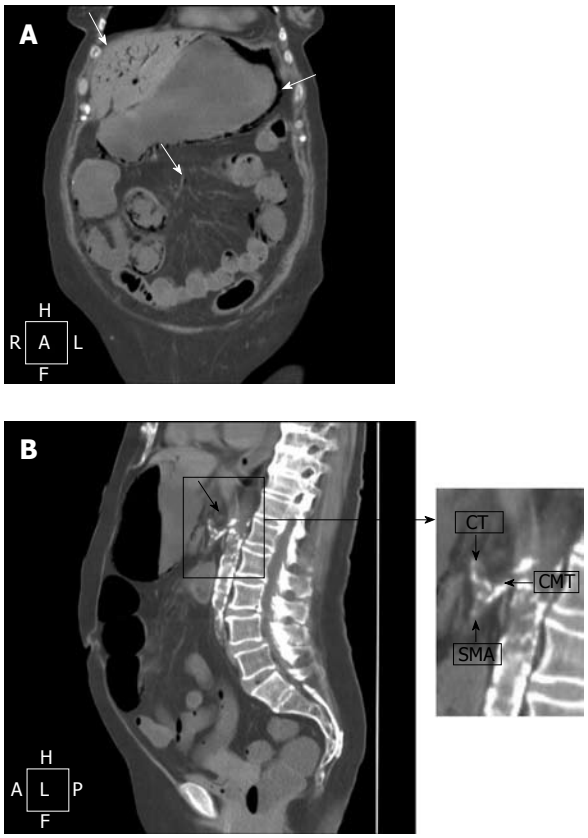

Figure 2 Computed tomography of the abdomen. A: Pneumatosis of the wall of stomach and small bowel (arrow in the right). Intraparenchimal air in ventral portions of the left liver and fourth segment (arrow in the left); air within the branches of the mesenteric vein (arrow in the centre); B:Thrombosis of the celiacomesenteric trunk (CMT) (arrow). In the detail: common origin of celiac trunk (CT) and superior mesenteric artery (SMA) from the CMT.

DISCUSSION

The celiac trunk and superior mesenteric artery supply the majority of the blood to the gastrointestinal tract. Usually, the celiac trunk is a short artery that arises from the anterior wall of the abdominal aorta at the level of the twelfth thoracic vertebra; it divides almost immediately into three branches: left gastric, splenic and common hepatic artery.

The left gastric artery courses upwards to the left toward the cardias, where it turns downward and, following the lesser gastric curvature, descends to the right toward the pylorus. The left gastric artery forms an arcading anastomotic loop with the right gastric artery, a branch of the common hepatic artery and, less frequently, of the gastroduodenal artery.

The splenic artery from its origin takes a short loop to the right and then runs along the cephalic border of the pancreas to supply the spleen. The splenic artery gives rise to several branches directed to the pancreas (dorsal pancreatic artery, arteria pancreatica magna, caudal pancreatic arteries) and to the stomach (left gastroepiploic artery, short gastric arteries).

The common hepatic artery arises on the right side of the celiac trunk and runs to the right reaching the first part of duodenum, where it gives rise to a branch called the gastroduodenal artery and continues into the hepatic artery proper (some authors don't utilise this definition, but they call this branch of celiac trunk the common hepatic artery "before" or "after" the origin of the gastroduodenal artery). At the porta hepatis it divides into right and left hepatic arteries. The common hepatic artery emerges partially or entirely from the superior mesenteric artery in approximately 18% of the population.

The celiac trunk supplies the liver, stomach, pancreas and superior part of the duodenum[1,2].

The superior mesenteric artery arises from the ante-

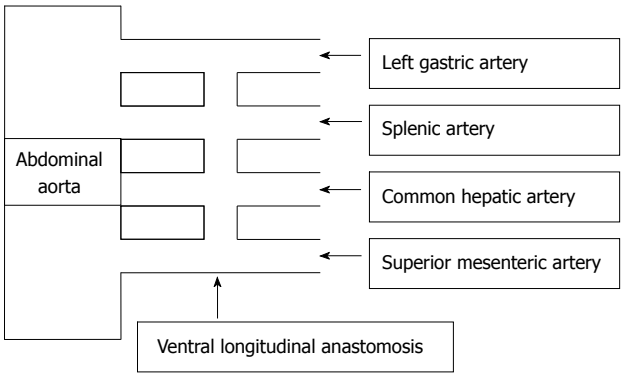

Figure 3 Primitive splanchnic vascularisation. Ventral longitudinal anastomosis (Lang's anastomosis) between four primitive splanchnic roots arising from the abdominal aorta.

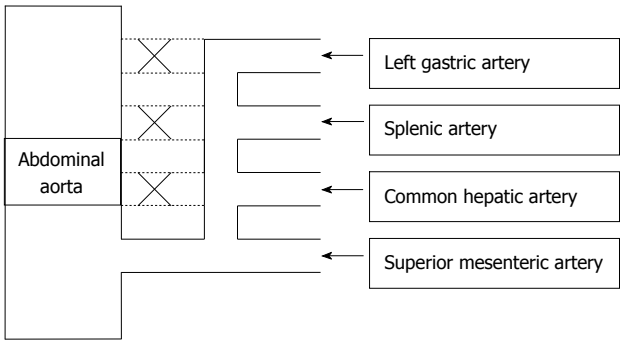

Figure 4 Celiacomesenteric trunk. Retention of the ventral longitudinal anastomosis higher than the fourth root keeps one or more celiac trunk branches with the superior mesenteric artery, disappearance of the first or fourth root causes a common celiacomesenteric trunk.

rior wall of the abdominal aorta at the level of the first lumbar vertebra. It runs down behind the head of the pancreas and ahead of the uncinate process of the pancreas and the third part of the duodenum. It descends anteriorly into the mesentery of the small intestine. Its right branches are inferior pancreatico-duodenal (this vessel forms an anastomosis with the superior pancreatico-duodenal artery, branch of the gastroduodenal artery), right colic, middle colic and ileo-colic; its left branches are 4-6 jejunal and 9-13 ileal arteries.

The superior mesenteric artery supplies the pancreas, duodenum (from the second to the fourth part), small bowel and colon from the caecum to the right half of the transverse colon[1,2].

Variations of the normal vascularisation described above may be caused by the retention or disappearance of the roots of the primitive arterial plexus, as indicated by Tandler[3] in 1908. The fetal digestive tube is supplied by four primitive splanchnic roots which arise from the abdominal aorta. There is a ventral longitudinal anastomosis (Lang's anastomosis) between these branches (Figure 3): the closure of the longitudinal anastomosis between the third and the fourth root and the disappearance of the central two roots lead to normal anatomy. Retention of the ventral longitudinal anastomosis higher than the fourth root keeps one or more celiac trunk branches with the superior mesenteric artery; disappearance of the first or fourth root causes a common celiacomesenteric trunk[2,4,5]. Moreover, the simple arboriform scheme of the gastroduodenal and hepatobiliary vasculature is profoundly altered by the growth of the liver and pancreas, and by the assumption of a curved form in the stomach and duodenum. These factors operate to complicate the branching of the coeliac axis and the superior mesenteric artery[6].

In our case, the celiacomesenteric trunk was formed by an anomalous separation of the ventral longitudinal anastomosis, with the common hepatic, left gastric and splenic arteries joining with the fourth root, as shown in Figure 4.

The celiacomesenteric trunk is one of the most striking among the different variations of the normal vascularisation of the gastro-intestinal tract[1,2,4-7]: it is found in 1%-2% of all anomalies involving the celiac axis[1,8-10]. Full comprehension of the topics as knowledge of the different anatomical variations of the arterial supply of the gallbladder, liver, stomach and colon is crucial in cholecystectomy, hepatobiliary and gastro-intestinal surgical procedures[6]. Without knowledge of the arterial architecture of the patient in this critical region, surgery may lead to a considerable risk of errors occasionally leading also to lethal complications[11].

Different classifications of these anatomical variations are present in the literature; however the most widely used are those indicated by Morita[12], Michels[13] and Olry *et al*[14]. The present case belongs to type I'b of Morita's classification and to type 6 of the Michels's classification.

The discovery of a celiacomesenteric trunk is often fortuitous during autoptic dissections[15-17] or can be accidentally detected by angiography or abdominal computed tomography scanning[5,8,10]. It can accompany different clinical situations, such as aneurysm[7,10,18], chronic occlusive disease [9,18], compression by abdominal aorta aneurysm or aortic dissection[18], celiac compression syndrome[9,19], but the large gastrointestinal infarction caused by thrombosis of the celiacomesenteric trunk, to our knowledge, has never been previously reported.

In the current case, the celiacomesenteric trunk arose at the level of the first lumbar vertebra and then, after a stretch of about one centimetre, it divided in two branches, superior (hepato-gastro-splenic trunk or celiac trunk) and inferior (superior mesenteric artery). The thrombosis of the origin of the celiacomesenteric trunk had a lethal effect on the patient because it caused the full stoppage of the splanchnic arterial supply and consequent ischemia. In fact, in this rare situation a single artery is the sole source of vascularisation of the supramesocolic organs and collateral flow is only possible from the inferior mesenteric, phrenic, oesophageal and retroperitoneal arteries[1]. Evidently, the condition of generalized atherosclerosis has prevented any collateral flow.

In conclusion, the present clinical report describes a

rare anomaly involving the celiac axis and, especially, the dramatic consequences related to the complete thrombosis of the celiacomesenteric trunk. This condition has important wide-ranging clinical implications because it compromises the blood supply of a large portion of the gastrointestinal tract and it may put at severe risk most of the abdominal viscera.

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Opioid/naloxone prolonged release combinations for opioid induced constipation

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Abstract

I read with great interest the recent article by Chen *et al* in a recent issue of your esteemed journal. The article is highly thought provoking. One emerging therapeutic alternative for opioid induced constipation is the emergence of opioid/naloxone prolonged release combinations. For instance, naloxone when administered in a 1:2 ratio with oxycodone reverses the inhibitory effect of oxycodone on the gastrointestinal tract. The advantage of oxycodone/naloxone prolonged release (OXN) is that while its anti-nociceptive efficacy is equivalent to that of oxycodone prolonged release (OXC), it significantly decreases the "Bowel Function Index" thereby ameliorating symptoms of opioid induced constipation to a large extent. Schutter *et al* in a recent study have reported a decrease in the bowel function index from 38.2 to 15.1. Similarly, Löwenstein *et al* in another recent study have reported that following a month of therapy, complete spontaneous bowel movements per week is increased from one in OXC therapy to three in OXN therapy.

Key words: Opiod naloxone; Cancer; Morphine; Carcinogenesis

TO THE EDITOR

I read with great interest the article by Chen *et al*[1] in a recent issue of your esteemed journal. The article is highly thought provoking. One emerging therapeutic alternative for opioid induced constipation is the emergence of opioid/naloxone prolonged release combinations.

For instance, naloxone when administered in a 1:2 ratio with oxycodone reverses the inhibitory effect of oxycodone on the gastrointestinal tract[2]. The advantage of oxycodone/naloxone prolonged release (OXN) is that while its anti-nociceptive efficacy is equivalent to that of oxycodone prolonged release (OXC), it significantly decreases the "Bowel Function Index" thereby ameliorating symptoms of opioid induced constipation to a large extent. Schutter *et al*[3] in a recent study have reported a decrease in the bowel function index from 38.2 to 15.1. Similarly, Löwenstein *et al*[4] in another recent study have reported that following a month of therapy, complete spontaneous bowel movements per week is increased from one in OXC therapy to three in OXN therapy.

In fact, the colonic transit time is reduced by almost two hours with OXN 20/10 mg combination therapy[5]. This is further affirmed by the fact that in patients receiving OXN therapy the mean laxative use is decreased by almost 20% while the stool consistency as measured by the "Bristol

Stool Form Scale" is improved from type 2 to type 5[6,7].

In addition, in a recent study, the quality of life was accentuated by 47% following OXN therapy for management of chronic severe neuropathic pain[8]. Similarly, a low mean Brief Pain Inventory Short Form "sleep interference" score is maintained with OXN therapy and is comparable to OXC therapy[9].

Clearly, OXN therapy is highly effective in mitigating the symptoms of opioid induced constipation and provides a safe and efficacious alternative to methylnaltrexone.

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Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

September 1-4, 2012
OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

September 15-16, 2012
Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech

October 19-24, 2012
American College of Gastroenterology 77th Annual Scientific Meeting and Postgraduate Course
Las Vegas, NV 89085, United States

November 3-4, 2012
Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

November 4-8, 2012
The Liver Meeting
San Francisco, CA 94101, United States

November 9-13, 2012
American Association for the Study of Liver Diseases
Boston, MA 02298, United States

December 1-4, 2012
Advances in Inflammatory Bowel Diseases
Hollywood, FL 33028, United States

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GENERAL INFORMATION

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Format

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-

ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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.00000 35706.28494.09]

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 1, 274 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

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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/index.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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