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^[1]Passed away on October 20, 2007

^[2]Passed away on June 14, 2008



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Emerging role of microRNAs in liver diseases

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Abstract

MicroRNAs are a class of small non-coding RNAs that are found in plants, animals, and some viruses. They modulate the gene function at the post-transcriptional level and act as a fine tuner of various processes, such as development, proliferation, cell signaling, and apoptosis. They are associated with different types and stages of cancer. Recent studies have shown the involvement of microRNAs in liver diseases caused by various factors, such as Hepatitis C, Hepatitis B, metabolic disorders, and by drug abuse. This review highlights the role of microRNAs in liver diseases and their potential use as therapeutic molecules.

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Key words: MicroRNA; Hepatitis; Fatty liver; Fibrosis; Cirrhosis

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INTRODUCTION

The recent discovery of several types of non-coding RNAs

revealed that the transcriptomes of higher eukaryotes are much more complex than originally anticipated. The non-coding RNAs, including microRNAs (miRNA), piwi interacting RNAs, small nucleolar RNAs, small interfering RNAs, long non-coding RNAs and antisense RNAs, have newly-discovered roles in the biology of health and diseases. These RNAs serve as modulators of genes involved in various biological pathways, such as development, cell differentiation, cell proliferation, cell death, chromosome modifications, virus pathogenesis, and oncogenesis^[1-3]. Among these RNAs, miRNAs are small RNA molecules of approximate 21 nucleotides that are present in most eukaryotes^[4]. Since the discovery of the first miRNA, lin-4, in 1993^[5], more than 500 miRNAs have been reported in the mammalian genome^[6].

It is postulated that about 1%-5% of genes in animals encode miRNAs, while 10%-30% of protein-coding genes are predicted miRNA targets^[7,8], and have a specific miRNA signature in normal or cancer cells^[9,10]. Although our understanding of the specific roles of miRNAs in cellular functions is only beginning, several studies have shown that miRNAs play a pivotal role in the most critical biological events, including development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction, organ development, hematopoietic lineage differentiation, host-viral interactions, and carcinogenesis^[4,11-13].

Overview of miRNAs

Despite the emerging critical role of miRNAs, the mechanism of their action is yet to be fully understood. The widely-known mode of gene regulation by miRNAs occurs at the post-transcriptional level by either specific inhibition of translation or induction of mRNA cleavage^[14]. The essential role of miRNAs in cellular physiology was elegantly shown in mice lacking the Dicer enzyme that is required for the processing of the precursor miRNAs into the mature form^[15].

Until now, the major focus of miRNA research was on their role in the cytoplasm, however recent reports indicate a diverse role of miRNAs, such as regulation of target genes by acting at 5' UTR^[16], regulation of DNA methylation^[17,18] and import of the mature miRNA into the nucleus, suggesting other functional modes^[19]. It is becoming clear that miRNAs not only regulate gene expression at the post-transcriptional level, but they are also capable of modifying chromatin (Figure 1).

In this review, we discuss the role of the newly emerging class of RNA, miRNAs, in different types of liver diseases.

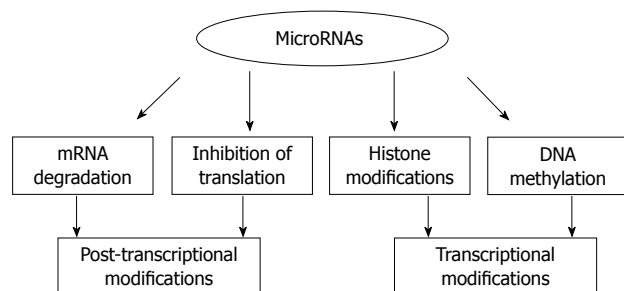


Figure 1 Various modes of gene regulation by miRNAs.

MiRNAs IN LIVER DISEASES

Improvements in the characterization and functional analysis techniques for miRNAs has not only uncovered their role in various cellular processes, but also revealed abnormal patterns of miRNA expression in various diseases, such as cancer^[20], viral infection^[21], inflammation^[22], diabetes^[23], cardiovascular^[24], and Alzheimer^[25]. In this review, we focus on the involvement of miRNAs in liver diseases caused by various factors, such as viral hepatitis, fatty liver due to alcohol abuse or metabolic syndrome, drug-induced liver disease, and by autoimmune processes (summarized in Table 1).

MiRNAs in host-viral response and viral hepatitis

Viruses are equipped with complex machinery to exploit the host biosynthetic pathways and to defend against host cellular responses^[26]. Recent studies have revealed the involvement of miRNA-mediated RNA-silencing pathways during viral-host cell interactions^[27]. However, little is known about the role of cellular miRNAs against viral infection in eukaryotic organisms. Interestingly, viruses not only exploit the cellular miRNAs, but also encode their own miRNAs, adding another layer of complexity^[28,29]. Therefore, it is important to study the abundance and distribution of miRNAs within the host cell during viral replication and latency, which will help us to understand the molecular regulation by the host cellular system as well as the simultaneous attempts by viruses to overcome the host defense.

Role of miRNAs in immune response: The interaction of parenchymal and immune cells plays a unique role in the liver in response to liver insults by viruses, bacteria, toxins, or antigens. There is compelling evidence that characterizes miRNAs as key regulators of innate and adaptive immune responses^[22,30] and, therefore, they might play a role in inflammatory, autoimmune, or viral diseases of the liver. The importance of miRNAs in the liver immune system is highlighted by the fact that mice lacking Dicer 1 function in the liver were unable to produce mature miRNA and showed progressive hepatocyte damage, apoptosis, and portal inflammation^[31].

The innate immune response is the first line of defense against noxious agents and is mediated by adaptors, such as the toll like receptors (TLRs), which recognize specific molecular patterns. Signals triggered by TLRs are involved in most liver diseases (from viral to inflam-

Table 1 Expression of miRNAs in liver diseases

Liver	Upregulated	Downregulated	Ref.
HCV	miR-215, miR-16, miR-199, miR-155, miR-146	miR-122, miR-320, miR-191	[53]
HBV	miR-181a, miR-200b and miR-146a	miR-15a	[56]
Drug overdose	miR-711 (liver), Plasma: miR-122 miR-15a, miR-21, miR-101b, miR-148a and miR-192	miR-29b(liver), miR-710 (plasma)	[62]
NAFLD	miR-122, miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221 and miR-200a	miR-29c, miR-451, miR-21	[69,70]
ALD	miR-212, miR-320, miR-486, miR-705, and miR-1224	miR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322	[71,72]
PBC	miR-328 and miR-299	miR-122a and miR-26a	[73]
Liver fibrosis	miR-27a, miR-27b, miR-30,	miR-9, miR-721 and miR-301	[80]
HCC	miR-18, miR-21, miR-221, miR-222, miR-224, miR-373 and miR-301	miR-122, miR-125, miR-130a, miR-150, miR-199, miR-200 and let-7 family members	[81,90-95]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease; ALD: Alcoholic liver disease; PBC: Primary biliary cirrhosis; HCC: Hepatocellular carcinoma.

matory conditions) as well as in liver regeneration^[32]. Several miRNAs (such as miR-155 or miR-146a/b) have been implicated in the regulation of TLR-induced signaling and might associated with liver pathophysiology. In particular, miR-155 was upregulated in monocytes and macrophages both upon TLR2, TLR3, TLR4, and TLR9 stimulation and after exposure to cytokines such as tumor necrosis factor (TNF)- α and interferons (IFN)^[33-35]. The exact function of this miRNA in macrophages is not completely clear, but it might exert a positive regulation on TNF- α release, through targeting genes involved in nuclear factor- κ B (NF- κ B) signaling or enhancing TNF- α translation^[34]. miR-146 is also upregulated in response to TLR2, TLR4, and TLR5 stimulation in monocytes^[33]. This miRNA seems to decrease the release of inflammatory mediators, such as interleukin (IL)-1 β or IL-8^[36], possibly by downregulating IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6, and act as a negative regulator of TLR signaling^[33]. Most of the studies describing the importance of these miRNAs in the innate immune response have been performed in macrophages or monocytes *in vitro*, and therefore the elucidation of specific miRNA functions *in vivo* in Kupffer cells, the resident liver macrophage, still awaits confirmation.

Neutrophil leukocyte infiltration in the liver is also a part of the innate immune response, which is common to liver injury, hepatic stress, or systemic inflammation signals^[37]. Regulators of granulocyte function and activation, such as miR-223, might also be able to play a role in liver

disease. Several reports have shown that the transcription factor C/EBP α binds to the miR-223 promoter and enhances its expression^[38-40]. Given the importance of antigen presentation in the liver, the involvement of miRNAs in this function could be also of interest. In line with this, Hashimi *et al.*^[41] have recently shown that inhibition of both miR-21 and miR-34a in monocyte-derived dendritic cells led to a decrease in endocytic capacity and altered differentiation.

Apart from their role in the innate immune responses, miRNAs are associated with many aspects of both normal and abnormal immune adaptive responses, making them likely actors in autoimmune liver diseases or, particularly, viral hepatitis^[22,30]. Virtually every T and B cell type seems to be associated with a different miRNA expression profile, and therefore an interesting avenue of research is to identify the miRNAs signature associated with specific liver diseases. Regarding specific miRNA functions, miR-181 has been described as a key quantitative regulator of T cell response to antigens^[42]. MiR-150 plays a crucial role in B cell differentiation because its over-expression results in a selective defect in B cell development that blocks the transition from pro-B cell to pre-B cell^[43]. Of note, miR-155 is implicated in both T and B cell function and differentiation promoting T helper 1 vs type 2 differentiation^[44,45] and is essential in the production of antigen-specific antibodies^[46]. These data highlight the importance of this miRNA as a central actor in immune regulation.

Hepatitis C virus (HCV) infection: HCV is an enveloped RNA virus of the Flavivirus family, and is capable of causing both acute and chronic hepatitis in humans by infecting liver cells. It is a major cause of chronic liver disease, with about 170 million people infected worldwide^[47]. Up to 70% of patients will have persistent infection after inoculation, making it a fatal disease. The disease varies widely, from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma (HCC)^[47]. Since the discovery of HCV, the treatment of hepatitis C has considerably improved by the use of pegylated interferons with ribavirin; however, more than 50% of people still do not respond to current medication and thus there is need for better drug therapy.

Recent studies have shown the involvement of miRNAs in the regulation of HCV infection. MiR-122 is first identified liver-specific cellular miRNA, which has been shown to enhance the replication of HCV by targeting the viral 5' non-coding region^[48]. It appears that HCV replication is associated with an increase in expression of cholesterol biosynthesis genes that are regulated by miR-122 and hence is considered as a potential target for antiviral intervention^[49].

To date, there are some controversies around the role of miR-122 in HCV infection. Recently, Henke *et al.*^[50] showed that miR-122 stimulates HCV translation by enhancing the association of ribosomes with the viral RNA. The findings describing the role of miR-122 in HCV replication are of special interest, not only because of its novel mode of action, but because they also provide the first evidence of miRNAs linked to

infectious disease. The development of therapeutic products to inhibit the miR-122 is believed to be an attractive approach to treat HCV patients.

By contrast, Sarasin-Filipowicz *et al.*^[51] found that liver biopsies from chronic hepatitis C patients undergoing IFN therapy revealed no correlation between miR-122 expression and viral load. Moreover, they found markedly decreased miR-122 levels in people who did not have any virological response during later IFN therapy. These reports indicate that the role of miR-122 in HCV viral replication is still controversial and awaits future research.

While some host miRNAs are beneficial for the virus, others inhibit viral replication. Studies by Pedersen *et al.*^[52] demonstrated that IFN- β stimulation, an antiviral cytokine, induces numerous cellular miRNAs, specifically eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448) that have sequence-predicted targets within the HCV genomic RNA. Overexpression of these miRNAs in infected liver cells considerably attenuated viral replication. From this report it seems that host miRNAs have evolved to target viral genes and inhibit their replication, and thus might represent part of the host antiviral immune response. Very recently, Peng *et al.*^[53] carried out a computational study of hepatitis C virus associated miRNAs-mRNA regulatory modules in human livers. They found differential profiles of cellular miRNAs that target the genes involved in chemokine (16 genes such as *CXCL12* *etc.*), B cell receptor, PTEN (13 genes), IL-6 ERK/ MAPK (18 genes; Ras, Erk3 and STAT3 *etc.*) and JAK/STAT signaling pathways, suggesting a critical role of miRNAs in the replication, propagation, and latency of virus in the host cell. Specifically, they found that miR-122, miR-320, and miR-191 were downregulated, whereas miR-215, miR-16, miR-26, miR-130, miR-199 and miR-155 were upregulated. These findings suggest that miRNAs have the potential to become novel drug targets in virally induced infectious or malignant diseases.

Hepatitis B virus (HBV) infection: HBV infection is a global problem and it can cause acute or chronic hepatitis B, liver cirrhosis, and, in some instances HCC^[54]. The available therapy is only partially effective against the virus and development of better therapy remains an important issue. Little is known about the involvement of miRs during HBV infection. Considering the fact that cellular miRNAs play an important role in viral pathogenesis, it is likely that they have a role in HBV infection.

A differential miRNAs expression pattern was found in the livers of HBV and HCV infected individuals with hepatocellular cancer^[55]. A total of nineteen miRNAs were clearly differentiated between HBV and HCV groups, out of which thirteen miRNAs were downregulated in the HCV group, whereas six showed a decreased expression in the HBV group. Some of the differentially regulated miRNAs between the HCV and HBV groups were miR-190, miR-134, miR-151, miR-193, miR-211, and miR-20. Interestingly, in the same study, it was shown that pathway analysis of targeted genes using infection-associated miRNAs could differentiate the genes into two groups. For instance,

in HBV-infected livers, pathways related to cell death, DNA damage, recombination, and signal transduction were activated, and those related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism were activated in HCV-infected livers^[55].

In a second study, the profiling of cellular miRNAs of a stable HBV expressing cell line HepG2.2.15 and its parent cell line HepG2, showed that eighteen miRNAs were differentially expressed between the two cell lines^[56]. Of them, eleven were upregulated and seven were downregulated. For instance, miR-181a, miR-200b, and miR-146a were found to be upregulated and miR-15a was downregulated. It remains to be evaluated if differentially regulated miRNAs could be exploited as potential biomarkers to differentiate between HCV and HBV in the initial stages of pathogenesis.

MiRNAs in drug-induced liver injury

Drug-induced liver injury is a serious clinical health problem and is the leading cause of drugs being removed from the market^[57]. Acetaminophen overdose is the most common cause of fulminant liver failure. Many studies have been carried out to identify reliable and sensitive early blood markers for liver injury using high throughput technologies^[58]. However, these efforts have so far failed to yield markers that are superior compared to the existing aminotransferase-based markers, and the need for better and stable biomarkers persists. Increasing attention is being paid to circulating miRNAs as potential biomarkers because of their stability over enzymes or proteins^[59-61]. However, not much is known about the roles of miRNAs in drug-induced liver injury. A study by Wang *et al*^[62] showed differential miRNAs profiling in a mouse model of acetaminophen overdose. Significant differences were found in the levels of miRNAs in both liver tissues and plasma between control and acetaminophen overdosed animals. Using miRPortal (www.miRportal.net), pathways involving antigen processing and presentation, apoptosis, B cell receptor signaling pathways, cytokine-cytokine receptor interaction, and cell cycles were found to be potential targets of miRNAs that showed decreased levels in the liver after acetaminophen exposure. In contrast, pathways involved in various signaling transduction and cell-cell interactions, such as gap-junctions, focal adhesion, and MAPK signaling pathways were potentially affected by miRNAs with increased levels in the liver^[62].

Interestingly, it was observed that most miRNAs that were found to decrease in the liver exhibited increased levels in the plasma in overdosed mice. For instance, miR-122 and miR-192 showed higher levels in plasma, but they were decreased in tissue, whereas miR-710 and miR-711 were elevated in liver tissues; however, they displayed lower levels in plasma after acetaminophen overdose. Moreover, liver specific miRNAs, such as miR-122 and miR-192, showed dose and time-dependent changes in the plasma that paralleled serum aminotransferase levels and liver injury on histopathology. These findings suggest the potential of using specific circulating miRNAs as sensitive and informative biomarkers for drug-induced liver injury.

MiRNAs in non-alcoholic fatty liver disease (NAFLD)

NAFLD is the most common form of chronic liver disease worldwide and a major public health concern in the modern society^[63]. It is characterized by excess fat accumulation in liver, which ranges from simple steatosis to steatohepatitis, and cirrhosis in the absence of heavy alcohol consumption^[64]. The major risk factors for the development of NAFLD include metabolic syndrome, such as obesity, type 2 diabetes mellitus, and dyslipidemia^[65]. Although progress has been made in understanding the factors causing NAFLD, more needs to be done to dissect the underlying regulatory networks. Compelling evidences indicate the role of miRNAs in energy metabolism and liver functions^[66,67]. For instance, miR-143 has been shown to play a role in adipose differentiation^[68].

MiR-122, which was found to be upregulated in NAFLD, is known to regulate genes involved in fatty acid biosynthesis^[69], and administration of this miRNA antagonist into mice resulted in reduced levels of plasma cholesterol, increased hepatic fatty acid oxidation, and decreased synthesis of hepatic fatty acid and cholesterol.

Li *et al*^[70] reported that eight miRNAs (miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221 and miR-200a) were upregulated and three miRNAs (miR-29c, miR-451, miR-21) were downregulated in the livers of ob/ob mice. These findings suggested a connection between miRNAs and metabolic disorders, and the role of miRNA in NAFLD deserves further investigation.

Role of miRNAs in alcoholic liver disease (ALD)

ALD, alcoholic hepatitis, and cirrhosis represent a major segment of liver diseases worldwide. A recent report by Dolganiuc *et al*^[71] analyzed the miRNA expression profile in a murine model of ALD. Liver samples from mice fed an ethanol-containing diet (Lieber-DeCarli) showed features of alcoholic steatohepatitis and had an increased expression of miR-320, miR-486, miR-705, and miR-1224. On the other hand, decreased expression was found for several miRNAs, including miR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322. Furthermore, livers from mice with alcoholic and with non-alcoholic fatty liver shared common alterations in their miRNA profiles (such as upregulation of miR-705 and miR-1224). However, the functions and physiological roles of these miRNAs in ALD are yet to be determined. Tang *et al*^[72] have suggested a novel mechanism for ethanol-induced intestinal permeability. They found that ethanol intake resulted in the induction of miR-212, which in turn downregulated the Zonula occludens (ZOP) protein. The decrease in ZOP protein levels led to intestinal barrier disruption and thus increased intestinal permeability. The interest of this finding lies in the association of intestinal permeability with the development of ALD, therefore linking miR-212 with the pathogenesis of this disease.

Primary biliary cirrhosis (PBC)

Recent studies indicate that the role of miRNAs is yet to be explored in liver diseases associated with autoimmune

aggression. In PBC, altered hepatic miRNA expression was found^[73]. miRNA profiling of explanted livers from subjects with PBC revealed differential expression of thirty-five independent miRNAs compared to controls. Downregulation of miR-122a and miR-26a, and increased expression of miR-328 and miR-299, were confirmed by PCR in the PBC livers. The role of miRNAs in autoimmune hepatitis is yet to be studied.

Liver fibrosis

Chronic hepatitis contributes to liver fibrosis, which has been linked to fibrosis due to fibrin deposition by hepatic stellate cells (HSC)^[74]. The relationship between miRNAs and fibrosis has been shown in the context of specific diseases, such as diabetic kidney sclerosis^[75] or myocardial fibrosis^[76], and some of the mechanisms described could be involved in common pathways of fibrosis in other diseases. A recent report by Kato *et al*^[77] has described that transforming growth factor (TGF)- β activates Akt kinase by enhancing the expression of both miR-216a and miR-217. These miRNAs, in turn, target PTEN, an inhibitor of Akt activation that has also been described to play a role in hepatic stellate cell activation^[78]. Hepatic stellate cell activation has been identified as the major driver of liver fibrosis. Activation of HSC is associated with upregulation of various signaling pathways. In cirrhotic rat livers, HSC activation was associated with mitochondrial damage indicated by increased Bcl-2 and downregulated caspase-9 levels. The miRNA profile of the same rat HSC indicated upregulation of thirteen and downregulation of twenty-two, signaling pathways by miRNAs^[79]. In another in vitro study, overexpression of miR-27a and miR-27b resulted in reversal of the activated phenotype of stellate cells to a more quiescent phenotype with increased fat accumulation and decreased proliferation^[80]. The effects of miR-27a and miR-27b were mediated by regulation of the retinoid X receptor in the hepatic stellate cells.

Although there are only a few studies in the literature on miRNA genes and HCV-induced liver fibrosis, given the critical role of miRNA, its possible that they play a critical role in HCV infection, in the progression of fibrosis, and in the prediction of treatment response. Jiang *et al*^[81] showed increased miRNA expression in cirrhotic and hepatitis-positive liver samples and suggested that important changes in miRNA expression occur during the development of chronic viral hepatitis and cirrhosis.

HCC

HCC is the third-leading cause of death from cancer and the fifth most common malignancy worldwide^[82]. Liver cancer is a complex disease with exceptional heterogeneity in cause and outcome, involving epigenetic and chromosomal instability^[83] and abnormalities in the expression of both coding and non-coding genes, including miRNAs^[84,85]. The major etiologies of HCC include chronic liver disease due to chronic hepatitis B or hepatitis C virus infection, alcoholic steatohepatitis, metabolic disorders such as nonalcoholic steatohepatitis or insulin resistance, hereditary hemochromatosis, and

immune-related diseases such as PBC and autoimmune hepatitis^[86].

There are several studies in the literature showing specific miRNA signatures in HCC formation^[87-89] and progression that could be exploited as potential cancer biomarkers. Briefly, miR-18, miR-21, miR-221, miR-222, miR-224, miR-373, and miR-301 were reported to be upregulated^[81,90,91] whereas miR-122, miR-125, miR-130a, miR-150, miR-199, miR-200, and let-7 family members^[92-95] were found to be downregulated in HCC by most studies. These miRNAs target the genes involved in cell cycle and cell death regulation, including cyclin-dependent inhibitors *p27/CDKN1B* and *p57/CDKN1C*, or the PI3K antagonist phosphatase and tensin homolog (PTEN). Budhu *et al*^[96] defined twenty miRNAs, as a HCC metastasis signature, and showed that the twenty-miRNA-based signature was capable of predicting survival and recurrence of HCC in patients with multinodular or solitary tumors, including those with early-stage disease. miR-219-1, miR-207, and miR-338 were the most highly upregulated, whereas miR-34a, miR-30c-1, and miR-148a were highly downregulated in metastasis cases. Target Scan analysis revealed that *SPTBN2*, *GTF2H1*, *PSCD3*, *VAMP3*, and *SLC20A2* are the target genes which were affected by multiple miRNAs related to metastasis and might be the part of signaling pathways that significantly contribute to this phenotype. Moreover, this miRNA signature was an independent and significant predictor of patient prognosis when compared to other available clinical parameters^[96]. This study suggested that these twenty miRNAs can assist in HCC prognosis and might have clinical relevance. Further functional studies of these miRNAs could help to elucidate the mechanism leading to HCC metastasis. Deregulation of these twenty miRNAs in metastatic HCC implies that altered expression of their target genes might contribute to the development or recurrence of metastasis, and miR-122 is one example of this metastasis signature^[97].

THE POTENTIAL OF MiRNAs IN DIAGNOSTICS, DISEASE PROGNOSIS, AND THERAPY

Increasing evidence indicates that miRNAs play an important role in a wide range of liver diseases, ranging from cancers and viral hepatitis, to metabolic diseases. The unique expression profile of miRNAs in different types and at different stages of cancer, and in other diseases, suggest that these small molecules can be exploited as novel biomarkers for disease diagnostics and might present a new strategy for miRNA gene therapy.

Recently, Kota *et al*^[98] showed that administration of miR-26a, (which is under-expressed in HCC cells and downregulates cyclins D2 and E2) using adeno-associated virus (AAV) resulted in the inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity in a mouse model of HCC, suggesting the potential therapeutic use of miRNAs. This study

showed that anti-miRNA compounds could be delivered *in vivo* safely and with efficacy; thus opening the way for translation of these basic research findings to clinical applications. It is also important to consider other factors, because some miRNA genes such as miR-1 undergo methylation-mediated regulation in HCC cell lines^[99], suggesting a strong link between the DNA methylome and the miRNAome. In particular, there are some reports showing that the expression profiles of miRNA differ between malignant hepatocytes, malignant cholangiocytes, and benign liver cancer^[90], suggesting that miRNA profiling could be used as molecular diagnostic markers in liver disease.

CONCLUSION

It is increasingly evident that miRNAs play a novel and important role in regulation of gene expression. In recent years, research focusing on small molecules such as miRNAs has intensified to understand their role in health and disease. The excitement of exploring their regulatory potential will remain for years to come, given the observation that miRNAs could be ideal therapeutic targets for many diseases. We are at the beginning of understanding of the diverse roles of miRNAs in fine-tuning biological pathways, and in future years we will have a better picture of this complex regulatory mechanism. Presently, there is need for miRNAs-based diagnostics and gene therapy; however, these techniques are in their infancy.

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Acute pancreatitis in pregnancy

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Abstract

Acute pancreatitis (AP) is a rare event in pregnancy, occurring in approximately 3 in 10000 pregnancies. The spectrum of AP in pregnancy ranges from mild pancreatitis to serious pancreatitis associated with necrosis, abscesses, pseudocysts and multiple organ dysfunction syndromes. Pregnancy related hematological and biochemical alterations influence the interpretation of diagnostic tests and assessment of severity of AP. As in any other disease associated with pregnancy, AP is associated with greater concerns as it deals with two lives rather than just one as in the non-pregnant population. The recent advances in clinical gastroenterology have improved the early diagnosis and effective management of biliary pancreatitis. Diagnostic studies such as endoscopic ultrasound, magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiopancreatography and therapeutic modalities that include endoscopic sphincterotomy, biliary stenting, common bile duct stone extraction and laparoscopic cholecystectomy are major milestones in gastroenterology. When properly managed AP in pregnancy does not carry a dismal prognosis as in the past.

Key words: Acute pancreatitis; Pregnancy; Pancreatitis in pregnancy

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INTRODUCTION

Acute pancreatitis (AP) is a common problem with an annual incidence of 5 to 80 per 100000 of the general population. The incidence of AP in pregnancy varies and is approximately 1 in 1000 to 1 in 10000 births^[1]. The wide variation in the incidence is influenced by the prevalence of its most important etiological factor i.e. gallstone disease. While biliary pancreatitis complicated 1 in 3300 pregnancies at a large public hospital in Dallas, Texas^[2], in southern California 1 in 1500 women were affected^[3]. In a retrospective study from USA spanning 10 years Legro and Laifer^[4] identified 25 cases of AP in pregnancy. Eleven of these 25 patients were diagnosed in the first trimester of pregnancy when physicians had to clearly distinguish between hyperemesis gravidarum and AP. In another study 43 pregnant women out of 147197 were diagnosed with AP^[2]; 19% of these were diagnosed in the first trimester, 26% in the second, and 53% in the third trimester (one was postpartum), demonstrating that AP was more common with advancing gestational age, paralleling the frequency of gallstone diseases in pregnancy.

Older reviews of AP in pregnancy reported maternal and fetal mortality rates as high as 20% and 50% respectively^[2,5-9]. The above data from the pre-endoscopic retrograde cholangiopancreatography (ERCP), pre-laparoscopic cholecystectomy era are not valid anymore. Contemporary reports document a much improved outcome of AP in pregnancy, when the management of AP secondary to gallstones has undergone substantial changes^[10,11]. Hernandez *et al*^[11] in 2007, based on a single center experience spanning 10 years, reported 34 episodes of

AP with no maternal deaths and a fetal loss of only 4.7%. Date *et al*^[12] in 2008 compared conservative and surgical management of cholecystitis in pregnancy and noted no difference in fetal mortality (2.2% *vs* 1.2%, $P = 0.57$), and there was no maternal mortality. The major changes are the availability of many options in abdominal imaging and less invasive therapeutic options. In addition to abdominal ultrasound (US) we have endoscopic ultrasound (EUS), magnetic resonance cholangiopancreatography (MRCP) and ERCP. The introduction of laparoscopic cholecystectomy in 1986 is a milestone that has reduced the morbidity of surgical intervention by open abdominal surgery even in high-risk pregnant patients. Above all the safe applications of therapeutic ERCP, endoscopic sphincterotomy (ES) have permitted delaying cholecystectomy to safer periods in pregnancy or postpartum.

The etiological associations of AP during pregnancy are similar to those in the general population. AP in pregnancy is most often associated with gallstone disease or hypertriglyceridemia. Gallstones are the most common cause of AP during pregnancy responsible for more than 70% of cases^[2]. The incidence of gallstone related diseases including acute cholecystitis and biliary pancreatitis complicating pregnancy is 0.05%-0.8%^[13]. Even in patients who had prior cholecystectomy, a biliary etiology may exist. The prevalence of microlithiasis after cholecystectomy is 5%-10%^[14,15]. The pathogenesis of AP in gallstone disease is attributed to lodging or impaction of a stone or microlithiasis in the ampulla of Vater initiating premature activation of intracinar trypsinogen to trypsin.

AP OF BILIARY ETIOLOGY IN PREGNANCY

The prevalence rate of gallstones varies with ethnicity. Native American Indians, Mexicans, Latin Americans and Pima Indians all have a high incidence while the incidence is lower in Asia and Africa. Many studies have reported a high incidence of gallstones in the population from northern states of India^[16,17]. Gallbladder disease is strongly related to the metabolic syndrome, a problem that is growing in incidence all over the world^[18-21]. Rapid weight loss is a recently recognized factor for microlithiasis and gallstones^[22]. Although pregnancy itself is a risk factor, the risk increases with parity^[23]. Weight gain and hormonal changes predispose pregnant women to biliary sludge and gallstone formation^[24]. Identification of a biliary etiology for AP is important because as in the non-pregnant patient recurrence of AP episodes will occur in one-third to two-thirds of patients unless gallstones are removed^[10,11,25-27].

Pathogenesis of increased prevalence of gallstone in pregnancy

Cholesterol secretion in the hepatic bile increases in the second and third trimester compared to bile acids and phospholipids, leading to supersaturated bile; in addition, fasting and postprandial gallbladder volumes are greater, with reduced rate and volume of emptying.

This large residual volume of supersaturated bile in the gallbladder of the pregnant patient leads to the retention of cholesterol crystals and eventual gallstones. The formation of biliary sludge and stones is strongly associated with frequency and number of pregnancies^[25].

Up to 10% of patients develop stones or sludge over the course of each pregnancy, with obesity and increased serum leptin being risk factors^[28]. After delivery gallbladder motility becomes normal when sludge as well as stones may disappear^[26,27].

In evaluating pregnant patients with AP the four important questions to be answered are (1) does the patient have AP (establishing the diagnosis and ruling out other causes)? (2) if it is AP, what is the predicted severity? (3) is there a biliary etiology? and (4) what is the trimester of pregnancy?^[29] The answer to the last question determines the choice of imaging studies and mode of therapy.

In the management initial blood tests are done to establish the diagnosis of AP and to assess the severity. Serum amylase and lipase levels are reliable markers of AP during pregnancy. The serum lipase level is unchanged during pregnancy, and the amylase level is either normal or only mildly elevated^[30]. The alterations in blood chemistry in normal pregnancy do not hinder the assessment of severity. Elevation of serum alanine aminotransferase levels to > 3 times the upper limit of normal is a very sensitive biochemical marker of biliary pancreatitis^[31,32]. Any abnormality of liver enzymes and bilirubin as well as rapid change in the levels should suggest a biliary etiology.

IMAGING STUDIES

Abdominal ultrasound (AUS) with no radiation to the fetus is the initial imaging technique of choice to identify a biliary etiology. Gallstones as a potential cause of AP are identified by AUS in most cases^[33]. However, it is insensitive for the detection of common bile duct stones or sludge. When a common bile duct (CBD) stone is suspected based on AUS or biochemical abnormalities EUS, a semi-invasive procedure of the biliary tree is an accurate modality for detecting common bile duct stones^[34]. However EUS requires expensive equipment, intravenous sedation and technical expertise. EUS can be considered the best imaging study to evaluate CBD, although not for gallbladder stones. In expert hands small gallstones as well as sludge can be picked up by EUS, however it is operator dependent. EUS is appropriate prior to the consideration of therapeutic ERCP in patients where non-invasive imaging such as MRCP is not available, contraindicated or inconclusive. EUS has a high positive predictive value nearing 100% in detecting CBD stones and in many instances EUS is superior to MRCP^[35]. EUS entails no radiation exposure and is extremely safe apart from a minimal sedation related risk. If a common bile duct stone is detected, an ERCP with sphincterotomy can be performed following the EUS study during the same sedation.

Magnetic resonance imaging (MRI) and MRCP provide multi-planar large field of view images of the

body with excellent soft-tissue contrast and images of bilio-pancreatic duct systems. MRCP does not require any contrast injections and has no risk of renal injury. MRCP is a preferred method of evaluating CBD in many clinical situations. There is paucity of data on the safety of MRI in the first trimester of pregnancy^[36-38]. Some authors have raised concerns of thermal injury to the fetus in first trimester^[39,40].

According to the Safety Committee of the Society for Magnetic Resonance Imaging^[41], MR procedures are indicated in pregnant women if other non-ionizing forms of diagnostic imaging studies are inadequate, or if the examination provides important information that would otherwise require exposure to ionizing radiation [i.e. X-Ray computerized tomography (CT), *etc.*]. Gallstone pancreatitis is generally associated with small gallbladder stones and sludge^[42]. Small ductal stones in particular, located in the distal CBD could be missed by MRCP^[43]. Claustrophobia remains the major barrier in the use of MRCP and MRI.

CT scan of the abdomen is the most commonly used imaging modality in diagnosing and later on in assessing severity of AP among adults. It is not recommended in pregnant patients because of the fear of radiation exposure to the fetus^[44]. In general CT is not the preferred modality of imaging in all trimesters of pregnancy in view of a small radiation risk to the fetus.

ERCP solely as a diagnostic study has lost its value because of the risks of radiation, incidence of AP post procedure, and the availability of safer procedures such as EUS or MRCP. ERCP increases the risk of complications and death from 5% to 10% and 0.1% to 0.2% respectively^[45]. However, the clinical usefulness of therapeutic ERCP when indicated is unchallenged. Persistent biliary obstruction worsens the outcome, increases the severity of AP, and predisposes to bacterial cholangitis. ERCP along with ES helps to extract impacted gallstones and drain infected bile in severe AP^[46]. Several reports have shown that ERCP can be carried out successfully in the management of symptomatic choledocholithiasis in pregnancy^[47,48]. A major concern of this procedure is harmful ionizing radiation to the fetus. Tham *et al*^[49] reported their experience with ERCP in pregnancy (15 patients over 5 years) with fetal dose radiation measurement. The fetal radiation dose could be reduced to a level less than that considered teratogenic. Kahaleh *et al*^[50] looked at 17 ERCPs performed in pregnant women between January 1995 and August 2003. They reported a mean gestational age of 18.6 wk, mean fluoroscopy time of 14 s and an estimated fetal radiation exposure of 40 mrad. By limiting fluoroscopy time, shielding the pelvis and fetus with lead and avoiding direct X-ray films, the fetal radiation dose can be reduced to far below the maximum permissible doses. Performing MRCP or EUS before ERCP helps to identify patients who require therapeutic ERCP thus reducing the number of ERCP^[51].

MANAGEMENT

Several recommendations below are mostly based on

expert opinion only and not confirmed by double blind/randomized controlled trials. The difficulties in performing such studies in critically ill pregnant patients are obvious.

Nutrition

Although successful outcomes can be achieved in obstetric patients requiring parenteral nutrition, the frequency of maternal complications secondary to centrally inserted central venous catheters (TPN) is greater than that reported in non-pregnant patients^[52]. Peripherally inserted central catheters may be preferable when parenteral nutrition is required during pregnancy. Enteral nutrition by naso-jejunal feeding is preferable to TPN^[11] in patients with severe AP. Enteral nutrition is physiological, helps the gut flora maintain the gut mucosal immunity, reduced translocation of bacteria, while simultaneously avoiding all the risks of TPN.

Antibiotics

The topic of prophylactic use of antibiotics is very controversial and the choice of antibiotic in pregnancy is difficult. However, in suspected cholangitis there is no controversy with regards to the need for appropriate antibiotic therapy. Patients with mild AP, normal CBD size with no evidence for cholangitis do not need antibiotics. In a pregnant patient there are concerns with regard to the antibiotic being transplacentally transferred to the fetus with a risk of teratogenicity. Metronidazole passes freely across the placenta. However, recent studies do not show any association with an increased risk of teratogenic effects with metronidazole^[53,54]. Imipenem (N-formimidoyl thienamycin), belonging to the carbapenem class of antibiotics, has a broad spectrum of activity. It is currently classified as a category C in terms of its risk to the fetus. Although limited animal studies have shown no teratogenic risk or adverse fetal effects, data in humans are not available^[55]. Quinolones have been classified as category C because adverse effects have been noted in some animal studies. However, there are no adequate studies in humans; the benefits may outweigh the risks. Ampicillin-sulbactam and piperacillin/tazobactam are classified as category B with no evidence of risk in humans. Regardless of initial drug regimen, therapy should be modified to reflect the organisms recovered in blood cultures and the clinical status of the patient.

Management of underlying cause

Management of gallstones: In a pregnant woman with gallstones and CBD stones a major decision is on choice of procedure to clear the CBD of stones. The second decision is on timing and approach to cholecystectomy^[56]. Factors which influence the decision include the trimester of pregnancy, presence or absence of CBD dilatation, cholangitis, and the severity of AP. AP patients with gallstones need to be evaluated for early cholecystectomy to prevent recurrence of AP later on in the pregnancy when it could be more serious and dangerous^[25-27]. It is a well respected surgical concept that the second trimester is the best period for surgery since during this period organogenesis is complete and the uterus

is not big enough to obliterate the surgical view for laparoscopic approach. It has also been recognized that cholecystectomy during the second trimester is safe for both the mother and the fetus^[10,12,57].

Laparoscopic cholecystectomy in pregnant women offers all of the advantages of laparoscopic surgery in non-pregnant patients - reduced hospital stay, decreased narcotic use and a quick return to a regular diet compared to open surgery in pregnant women^[58]. In the second trimester the gravid uterus does not interfere with visualization of the operative field. The indications for surgery in pregnancy are severity of symptoms, obstructive jaundice, acute cholecystitis intractable to medical treatment and peritonitis.

Four retrospective studies comparing open cholecystectomy *vs* laparoscopic cholecystectomy did not find any significant difference in maternal or fetal outcomes^[12]. Gouldman *et al*^[59] reviewed the available world literature on laparoscopic cholecystectomy in pregnancy and found 107 patients who had cholecystectomy during pregnancy. Most had been performed in the second trimester, with 10 and 16 patients in the first and third trimesters, respectively. Premature labor was rare, with only 2 of the 16 reported patients (12.5%) in the third trimester developing preterm labor, and these were successfully treated with tocolytics. Overall results were good with excellent maternal (100%) and fetal (96%) survival. There is a recent view that states when surgical intervention is warranted, laparoscopic cholecystectomy can be safely performed in any trimester^[60] but it is a minority view. Performance of cholecystectomy is desirable in the second trimester as organogenesis is complete, and spontaneous abortions are less frequent than in the first trimester^[61].

ERCP with sphincterotomy and clearance of bile duct stones is indicated in patients with severe AP, with cholangitis, with strong evidence of persistent biliary obstruction, and in those who are post cholecystectomy as well as patients who are poor candidates for surgical therapy^[25]. Pregnant women in the first and third trimester who are not ideal candidates for cholecystectomy fall in the last category. Biliary sphincterotomy rather than cholecystectomy may be appropriate when CBD stones are detected and cholecystectomy has to be delayed because of pregnancy. The effectiveness of ES in preventing further episodes of biliary pancreatitis, as an alternative to cholecystectomy in high risk patients has been demonstrated^[48,62-69]. The indication for ERCP in patients with severe pancreatitis without significant cholestasis is controversial. At this time there is no evidence that therapeutic ERCP is required in all patients with biliary sludge during pregnancy.

The role of therapeutic ES in the management of pregnant patients with AP without CBD stones continues to be controversial^[70]. Some advocate biliary stent placement rather than performing sphincterotomy and stone extraction and therefore, eliminating complications that accompany sphincterotomy. Farca *et al*^[71] placed 10-French biliary stents without sphincterotomy in 10 patients, all of whom had uncomplicated pregnancies with normal deliveries. All underwent repeat ERCP with stent extraction and sphincterotomy post-partum and 8 had stones

extracted. In 2 patients, the stent remained in place for 7 and 8 mo, respectively, without the development of occlusion and or cholangitis. However, stenting carries risks of stent occlusion and cholangitis and the need for a second procedure.

Hyperlipidemic pancreatitis: Hypertriglyceridemia is the second most common cause of AP, when the serum triglyceride is > 1000 mg/dL. In the third trimester of pregnancy, there is a three-fold rise in serum triglyceride levels^[72]. This is thought to be due to estrogen-induced increases in triglyceride synthesis and very low-density lipoprotein secretion^[72]. Hypertriglyceridemia may be more severe in persons with familial hyperlipidemia, predisposing them to develop pancreatitis^[73]. Rarer causes of AP that need to be considered in the differential diagnosis are hyperemesis during the first trimester; hyperparathyroidism; preeclampsia; and genetic mutations^[74-76] and acute fatty liver of pregnancy. AP can also complicate the course of thrombotic thrombocytopenic purpura during pregnancy^[77] and pregnancy induced hypertension^[78]. Medication and alcoholism are extremely rare causes of AP in pregnancy.

No formal recommendations exist for gestational hypertriglyceridemia treatment in pregnancy at present. Treatment of hyperlipidemic AP is mostly supportive. These treatments include low fat diet^[79,80], antihyperlipidemic therapy^[79,80], insulin^[79-81] (to increase lipoprotein lipase activity), heparin^[79-81] (to increase lipoprotein lipase activity), and even plasmapheresis^[79,82].

CONCLUSION

AP in pregnancy remains a challenging clinical problem to manage, with a relatively limited but expanding evidence base. Among the various etiological factors for AP in pregnancy, gallstone disease is the most common one. Abdominal ultrasound, CT scan, EUS and MRCP are the available imaging studies in diagnosing a biliary etiology for AP. Potential radiation to the fetus is a major disadvantage with CT scan, restricting their use substantially. Diagnostic ERCP is to be avoided whenever possible owing to the associated risks including bleeding, perforation, pancreatitis, fetal radiation, while abdominal ultrasound, MRCP and EUS do not carry these risks. The general management of AP in pregnancy is supportive and includes hospitalization, intravenous fluids, analgesia, and bowel rest. Laparoscopic cholecystectomy is ideally performed in the second trimester when the risk to fetus is the least and only limited technical problems exist as a result of an enlarging uterus. Whenever laparoscopic cholecystectomy is not feasible and the index of suspicion for a stone in the CBD is high based on AUS, MRCP or by EUS, ES or stenting serves to prevent recurrence of AP and allows postponement of laparoscopic cholecystectomy to a more suitable period. Hyperlipidemic pancreatitis and AP due to other etiologies are rare. The outcome of pregnant patients with AP has substantially improved with technical advances in imaging and therapeutic endoscopy.

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Hepatitis C virus in Pakistan: A systematic review of prevalence, genotypes and risk factors

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Abstract

In Pakistan more than 10 million people are living with Hepatitis C virus (HCV), with high morbidity and mortality. This article reviews the prevalence, genotypes and factors associated with HCV infection in the Pakistani population. A literature search was performed by using the keywords; HCV prevalence, genotypes and risk factors in a Pakistani population, in Pubmed, PakMediNet and Google scholar. Ninety-one different studies dating from 1994 to May 2009 were included in this study, and weighted mean and standard error of each population group was calculated. Percentage prevalence of HCV was $4.95\% \pm 0.53\%$ in the general adult population, $1.72\% \pm 0.24\%$ in the pediatric population and $3.64\% \pm 0.31\%$ in a young population applying for recruitment, whereas a very high $57\% \pm 17.7\%$ prevalence was observed in injecting drug users and $48.67\% \pm 1.75\%$ in a multi-transfused population. Most prevalent genotype of HCV was 3a. HCV prevalence was moderate in the general population but very high in injecting drug users and multi-transfused populations. This data suggests that the major contributing factors towards increased HCV prevalence include unchecked blood transfusions and reuse of injection syringes. Awareness programs are required to decrease the future burden of HCV in the Pakistani population.

INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989 as the major causative agent of non-A, non-B hepatitis^[1]. It belongs to the *Flaviviridae* family and is a plus-stranded RNA virus^[2]. About 200 million people are infected with HCV worldwide, which covers about 3.3% of the world's population^[3,4]. HCV infection leads to chronic hepatitis in 50% to 80% of individuals^[5]. It was estimated by the WHO in 2004 that the annual deaths due to liver cancer caused by HCV and cirrhosis were 308 000 and 785 000 respectively^[6].

Pakistan is a developing country of 170 million people with low health and educational standards. According to the human development index of the United Nations, it was ranked 134th out of 174 countries^[7]. In Pakistan 10 million people are presumed to be infected with HCV^[8]. Public health authorities are creating awareness about hepatitis through print and electronic media^[9], but still tremendous efforts are required to increase the awareness regarding various risk factors involved in HCV transmission. In developing countries, due to non-implementation of international standards regarding blood transfusion, reuse of needles for ear and nose piercing, reuse of syringes, injecting drug users, tattooing, shaving from barbers, unsterilized dental and surgical instruments are the main source of transmission of HCV. This article briefly presents the prevalence, genotypes and risk factors associated with HCV transmission in the Pakistani population.

LITERATURE SEARCH

Articles were searched for in Pubmed, Google scholar and PakMediNet (for non-indexed Pakistani journals),

by using the key words; HCV in Pakistan, prevalence of HCV in Pakistan, epidemiological patterns of HCV in Pakistan, HCV in multi-transfused Pakistani population, HCV in general Pakistani population, HCV in Pakistani injecting drug users (IDUs) population, HCV in Pakistani health care workers, sexual transmission of HCV, injection use in Pakistan, blood banks/transfusion in Pakistan, awareness about HCV in Pakistani population and HCV genotypes in Pakistan. Inclusion criteria entailed the studies demonstrating the prevalence, genotypes and risk factors of HCV in the Pakistani population while studies with incomplete references were excluded. Two hundred and eighty-one different articles/abstracts/reports were obtained from the literature search, out of which 91, published from 1994 to May 2009, were included in this study.

ANALYSIS

Table 1 includes various reports showing the percentage prevalence of HCV in different groups. Weighted mean of each population was calculated by using the formula:

$$\bar{x} = \frac{\sum_{i=1}^n \omega_i x_i}{\sum_{i=1}^n \omega_i}$$

Standard error of mean was calculated by using the formula:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Results of each population group are presented in the form of mean $\pm$ SE with 95% confidence interval.

HCV PREVALENCE IN VARIOUS GROUPS

General population

Ten different studies showed that the percent prevalence of HCV in the general adult population was $4.95\% \pm 0.53\%$ ^[10-19], while six studies showed a percent prevalence of $1.72\% \pm 0.24\%$ in the pediatric population^[20-25]. In Pakistan, military recruits are screened for HCV before induction; six different studies showed a percent prevalence of $3.64\% \pm 0.31\%$ in candidates for military recruitment^[26-31]. About 5% of infants obtained HCV infection transmitted from a mother carrying both HCV antibody and HCV RNA^[32]; four studies in pregnant women showed the percent prevalence to be $4.54\% \pm 3.5\%$ ^[33-36]. Volunteer blood donors are the healthiest population in a community and HCV prevalence in these individuals is a true reflector of a general population's health^[9]; twelve different studies showed the percent prevalence to be $3.78\% \pm 0.41\%$ in blood donors^[17,37-47].

IDUs

It was estimated that there were about 5 million drug users in Pakistan, out of which 15% were regular IDUs^[48]. There has been an increased shift among addicts from inhalatory to injectable drugs due to decrease in quality and availability of heroin (common inhalatory drug used in Afghanistan and Pakistan)^[49]. The effect of injecting drugs is more intense and satisfying, and young drug users who switch over to injectables usually adopt it as the

main route of their drug administration^[50]. Approximately 50% of IDUs reported by Altaf *et al*^[51] in 2007 were in a treatment program; the majority of them wanted to get rid of their addiction but could not do so due to non availability or high charges by rehabilitation centers. The main reason for relapse was the economic crisis which most addicts suffer because the rehabilitation centers are not involved in the development of vocational skills among addicts. Four different reports showed a high $57\% \pm 17.7\%$ prevalence of HCV among the IDUs^[52-55].

Multi-transfused population

Thalassemic and hemophilic patients require life-long blood transfusions, so it is necessary to obtain screened blood from a reputable source, because the multi-transfused population is more prone to blood-borne pathogens. Arif *et al*^[56] reported that only 15.8% of parents of thalassemic children knew the importance of blood screening. Six different reports showed an HCV percent prevalence of $48.67\% \pm 1.75\%$ among the thalassemic and hemophilic population^[56-62].

Health care workers

Health care workers are at high risk of HCV infection because they are dealing with blood, blood-related products and instruments which may carry transmissible pathogens. Hamid *et al*^[63] reported that recapping of syringes is the key factor for receiving needle stick injuries in health care workers and that transmission of HCV by needle stick injury ranges from 2% to 10%. Two different reports showed an HCV percent prevalence of $5.2\% \pm 0.63\%$ in health care workers^[64,65].

Sexual transmission

It has been reported from the US that up to 20% of new HCV infections are due to sexual activity^[66]. Two different reports from the USA and Congo indicate low HCV prevalence among commercial sex workers^[67,68]. The main problems in Pakistan are illiteracy, lack of awareness about sexually transmitted diseases and low use of condoms among the sex workers. Saleem *et al*^[69] reported in 2005 that 17% of female sex workers, 3% of male sex workers and 4% of hijras (transgender men) consistently used condoms during the previous month; 67% of female sex workers were illiterate, 34% of female sex workers were suffering from sexually transmitted infections. Homosexual activities were very high among street children who are sexually victimized or indulge in such activities; later on they adopt commercial sex in order to raise their income. Condom usage among the male homosexual population was very low. Four different studies showed an interspousal percent prevalence of $17.24\% \pm 7.98\%$ ^[70-73].

RISK FACTORS

Unsafe and unnecessary needles

The reuse of syringes and needles was a major factor contributing towards increased HCV prevalence^[74,75]. It was reported that there are several small groups involved in recycling and repacking of used unsterilized

Table 1 Percentage prevalence of HCV among different communities in Pakistan

Population type	Author	Region	Methods	Population size	HCV (%)
General population	Luby <i>et al</i> ^[10] , 1997	Hafizabad	RIBA	309	6.50
	Parker <i>et al</i> ^[11] , 2001	Lahore	EIA	417	6.70
	Khokhar <i>et al</i> ^[12] , 2004	Islamabad	ELISA	47538	5.31
	Muhammad <i>et al</i> ^[13] , 2005	Buner	ELISA	16400	4.57
	Hashim <i>et al</i> ^[14] , 2005	Attock	ELISA	4552	4.00
	Zaman <i>et al</i> ^[15] , 2006	Bahawalpur	ICT	6815	4.41
	Alam <i>et al</i> ^[16] , 2006	Central Punjab	ELISA, ICT	2038	4.41
	Chaudhary <i>et al</i> ^[17] , 2007	Rawalpindi	MEIA	1428	2.52
	Hakim <i>et al</i> ^[18] , 2008	Karachi	ELISA, ICT, PCR	3820	5.20
	Tunveer <i>et al</i> ^[19] , 2008	Lahore	ICT	203	1.48
	Agboatwalla <i>et al</i> ^[20] , 1994	Karachi	ELISA	236	0.44
	Khan <i>et al</i> ^[21] , 1996	Lahore	EIA, RIBA	538	4.09
	Parker <i>et al</i> ^[22] , 1999	Lahore	ELISA	538	1.30
Pediatric population	Hyder <i>et al</i> ^[23] , 2001	Lahore	ELISA	171	0.58
	Jafri <i>et al</i> ^[24] , 2006	Karachi	ELISA	3533	1.60
	Aziz <i>et al</i> ^[25] , 2007	Karachi	EIA	380	1.40
	Ali <i>et al</i> ^[26] , 2002	Rawalpindi	ICT	5371	3.29
	Zakaria <i>et al</i> ^[27] , 2003	Karachi	ICT-ELISA	966	2.20
Recruitment	Masood <i>et al</i> ^[28] , 2005	Lahore	ELISA	4552	4
	Mirza <i>et al</i> ^[29] , 2006	Mardan	ELISA	15550	3.69
	Sharif <i>et al</i> ^[30] , 2006	Risalpur	ELISA	2558	3.40
	Alam <i>et al</i> ^[31] , 2006	Sargodha	unknown	2038	4.41
	Zafar <i>et al</i> ^[33] , 2001	Lahore	PCR	300	4
Pregnant women	Khokhar <i>et al</i> ^[34] , 2004	Islamabad	ELISA	503	4.80
	Jaffery <i>et al</i> ^[35] , 2005	Islamabad	ELISA, PCR	947	3.27
	Yousfani <i>et al</i> ^[36] , 2006	Hyderabad	ICT, ELISA	103	16.50
	Mujeeb <i>et al</i> ^[37] , 1996	Karachi	EIA	839	2.40
Blood donors	Bhatti <i>et al</i> ^[38] , 1996	Rawalpindi	EIA	760	4.80
	Mujeeb <i>et al</i> ^[39] , 2000	Karachi	ELISA	7047	2.40
	Ali <i>et al</i> ^[40] , 2003	Quetta	ELISA	1500	1.87
	Asif <i>et al</i> ^[41] , 2004	Islamabad	MEIA	3430	5.14
	Ahmad <i>et al</i> ^[42] , 2004	Peshawar	MEIA	4000	2.20
	Zaidi <i>et al</i> ^[43] , 2004	Peshawar	ELISA	49037	2.60
	Chaudry <i>et al</i> ^[44] , 2005	Lahore	ELISA	890	6.06
	Abdul Mujeeb <i>et al</i> ^[45] , 2006	Karachi	ELISA	7325	3.60
	Chaudhary <i>et al</i> ^[17] , 2007	Rawalpindi	MEIA	1428	2.52
	Sultan <i>et al</i> ^[46] , 2007	Different Areas	EIA	41498	4.99
	Khattak <i>et al</i> ^[47] , 2008	Different Areas	ELISA	103858	4
	Kuo <i>et al</i> ^[52] , 2006	Lahore	ELISA	351	88
	Achakzai <i>et al</i> ^[53] , 2007	Quetta	ELISA	50	60
	Altaf <i>et al</i> ^[54] , 2009	Karachi	ELISA	161	94
	Platt <i>et al</i> ^[55] , 2009	Abbottabad	ELISA	102	8
Thalassemic	Platt <i>et al</i> ^[55] , 2009	Rawalpindi	ELISA	302	17.30
	Bhatti <i>et al</i> ^[58] , 1995	Rawalpindi	ELISA	35	60
	Muhammad <i>et al</i> ^[59] , 2003	Peshawar	ELISA	80	36
	Shah <i>et al</i> ^[60] , 2005	NWFP	ELISA	250	57
	Hussain <i>et al</i> ^[61] , 2008	Islamabad/Peshawar	ELISA	180	41.70
Hemophilia	Hussain <i>et al</i> ^[62] , 2003	Peshawar	ELISA	40	25
	Malik <i>et al</i> ^[57] , 2006	Lahore	ELISA	100	56
Health care workers	Mujeeb <i>et al</i> ^[64] , 1998	Karachi	EIA	114	4.40
	Aziz <i>et al</i> ^[65] , 2002	Karachi	ELISA	250	5.60
Sexual/spousal	Irfan <i>et al</i> ^[70] , 2004	Islamabad	PCR	23	4.30
	Kumar <i>et al</i> ^[71] , 2004	Karachi	MEIA	50	18
	Khokher <i>et al</i> ^[72] , 2005	Islamabad	ELISA	227	4.40
	Qureshi <i>et al</i> ^[73] , 2007	Karachi	EIA/PCR	153	38

HCV: Hepatitis C virus; RIBA: Recombinant strip immunosorbent assay; ICT: Immunochromatographic test; ELISA: Enzyme linked immunosorbent assay; MEIA: Micro-enzyme immunoassay; EIA: Enzyme immunoassay; PCR: Polymerase chain reaction.

syringes, which were available in various drug stores. It was difficult for the public to differentiate between new sterilized syringes and recycled unsterilized syringes^[76]. Janjua *et al*^[77] reported that 68% of individuals received injections during the previous three months in Digri and Mirpur khas, two districts of Pakistan, out of which only 54% were from freshly opened syringes. The incidence

of sharing of injection equipment for the last injection was 8.5% in Hyderabad and 33.6% in Sukkur^[54].

In Pakistan, the number of estimated injections per person per year ranged from 8.2 to 13.6, which was the highest among developing countries, out of which 94.2% were unnecessary^[51]. Household members who received four injections per year were 11.4% more prone

to HCV infection than who did not receive injection^[78]. Khan *et al*^[74] reported that if both oral and injectable medicine were equally effective, 44% of the Pakistani population preferred injectable medicine.

In 2000, the WHO recommended that countries should implement strategies to change the behavior of health care workers and patients in order to decrease the over-use of injections, to ensure the practice of sterile syringes and needles, and to properly destroy sharp waste after use^[79]. It was reported that 59% of syringes were dumped into the general waste and not properly disposed of in the healthcare waste. Scavengers seeking valuable things from the waste are at high risk of receiving needle stick injuries from contaminated needles^[76].

Blood transfusion

People in developing countries are mostly anemic, and are more prone to traumatic injuries and obstetric complications. Blood transfusion in these situations is life-saving. If blood is not properly stored or is carrying blood-borne pathogens, then the situation becomes more complicated. According to the WHO office in Pakistan about 1.2 to 1.5 million transfusions are carried out annually in Pakistan^[80]. In 2000, Luby *et al*^[81] reported that 50% of blood banks in Karachi recruited paid donors, 25% of donations were from volunteer donors and only 23% of the blood banks screened for HCV while 29% of them were storing blood outside the WHO recommended temperature.

In developing countries, blood transfusion is still a problem due to lack of organized infrastructure, continuous supply of electricity, and properly trained and educated staff. In Pakistan the main source of blood donations are replacement donors and the majority of these are friends and relatives of the patient. These donors donate blood due to fear of death of a relative, chance of further complication of disease or under family pressure. These donors mostly hide their health conditions from their relatives. Selection of donor and their proper screening are key factors to ensure safe transfusion. Safe blood donors are those whose donations are repeatedly negative on screening^[17]. Sixty-six percent of Pakistanis are residents of rural areas where there is less access to blood transfusion services. To provide proper transfusion facilities to underdeveloped areas requires economic growth. Efforts are required to eliminate the transfusions from paid donors, to improve the safety of the blood supply^[81]. These conditions can be overcome by development of a fair and organized system of blood screening and transfusion.

Barbers

In third world countries like Pakistan, most of the barbers are illiterate and unaware of transmission of infectious agents through the repeated use of razors and scissors for different customers without sterilizing them first^[82]. Janjua and Nizamy reported that only 13% of the barber community knew that hepatitis is a liver disease and that it could be transmitted by contaminated razors; 11.4% of them were cleaning razors with antiseptic solution while 46% of them were re-using razors^[83]. Recent reports suggested that only 42% knew about hepatitis, 90% did

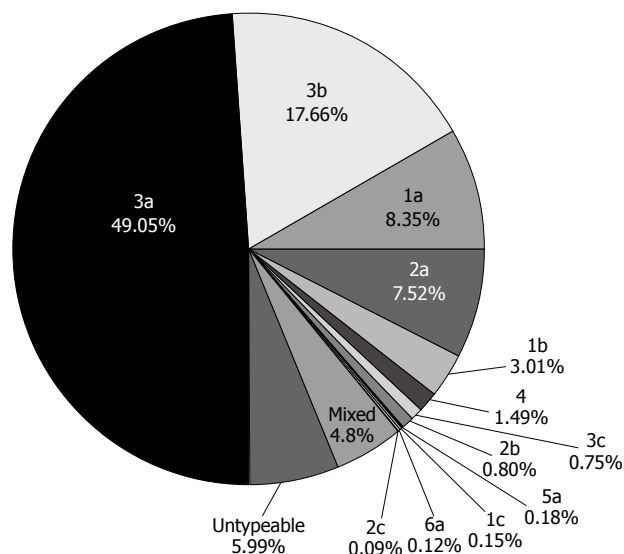


Figure 1 Hepatitis C virus (HCV) genotypes in Pakistan (2008)^[95].

not wash hands, 80% were not changing aprons and 66% were not changing towels after each customer. Circumcision is a very important religious procedure performed during early infancy both in rural and urban areas, and the barber community performing this procedure are mostly unaware of transmission of hepatitis by contaminated instruments^[84]. Bari *et al*^[85] identified the risk factors involved in transmission of HCV and reported that 70% and 48% of HCV patients had histories of facial and armpit shaving from barbers respectively.

Awareness

In the Pakistani population there was moderate knowledge about HCV infection whereas awareness about various HCV risk factors was very low^[31,86,87]. In a survey conducted at a family medicine clinic in Karachi, most of the participants had some educational background and were living in Karachi city. It was reported that 61% of participants believed that HCV was a viral disease, 49% believed that it could be transferred by needles and injections, 5.3% believed that it could be transmitted by ear and nose piercing, and 20.6% knew that it can cause cancer^[86]. Kuo *et al*^[52] reported in 2006 that HCV awareness was only 19% in the IDU population of Lahore and Quetta. Zuberi *et al*^[88] reported that knowledge about HCV infection was related to the educational background of the participants. Public awareness programs are required to decrease the future burden of HCV infection in the Pakistani population.

GENOTYPES

HCV is classified into eleven different genotypes, of which six are the major genotypes and these genotypes are further classified into many subtypes^[89,90]. In 1997 it was reported in a small study that 87% of the individuals in Pakistan had genotype 3^[91]. In 2004, a panel of 30 top gastroenterologists of the country met at a conference and reported that 75%-90% of HCV patients in Pakistan had genotype 3a^[8]. Qazi *et al*^[92] reported in 2006 that 71%

of patients had genotype 3 while only 10% had genotype 1. In 2007 it was reported that 81% of individuals had genotype 3 while only 9.5% had genotype 1^[93]. Hakim *et al*^[18] reported in 2008 that 51% of HCV patients had genotype 3a; 24% had 3a/3b co-infection and 16% had genotype 3b, while similar results were also reported by Afridi *et al*^[94] who stated that 50% of HCV patients had genotype 3a followed by 3b and 1a. The most detailed study was conducted by Idrees and Riazuddin in 2008, who performed genotyping of 3351 patients and reported that genotype 3a was the most prevalent genotype in Pakistan; their results are summarized in Figure 1^[95].

CONCLUSION

This study reviewed the seroprevalence of HCV among various population groups, along with risk factors and genotypes in Pakistan. HCV prevalence was observed in nearly 5% of the general population which is in parallel with the WHO estimates of HCV in Pakistan. High prevalence was observed in IDUs and the multi-transfused population, suggesting that the reuse of syringes was common among the injecting drug users, and that blood transfusions were not properly screened. Most prevalent genotype of HCV was 3a. The majority of HCV-positive patients had a history of facial and armpit shaving by barbers suggesting that the barbers shop was the key place for viral transmission. Condom usage was very low among the commercial sex workers and there was low awareness about sexually transmitted diseases amongst this group. In Pakistan, the number of estimated injections per person per year was very high because most Pakistanis think that injectable drugs are more efficacious than oral drugs. There was low awareness in people about the various risk factors associated with HCV transmission. Treatment of hepatitis is very expensive and is creating a huge burden on the country's economy. More emphasis should be given to the preventive measures of the disease in order to decrease the future health and economic burden; these include screened blood transfusions, proper sterilization techniques in clinics and hospitals, use of disposable syringes and razor blades. The government should take aggressive steps to create awareness among the general public by the use of media or by modifying the school syllabus.

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ORIGINAL ARTICLE

Shift work aggravates metabolic syndrome development among early-middle-aged males with elevated ALT

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Abstract

AIM: To examine whether shift work accelerates metabolic syndrome (MetS) development among early middle-aged males with elevated alanine aminotransferase (e-ALT).

METHODS: A retrospective, observational follow-up study on MetS development at a 5-year interval was conducted using health examination data. Nine hundred and ninety six male employees not fulfilling MetS criteria at screening were enrolled. Age, MetS-components, liver enzymes, serological markers for viral hepatitis, abdominal ultrasound, insulin resistance status, lifestyles, and workplace factors were analyzed.

RESULTS: The prevalence of elevated serum ALT (> 40 U/L, e-ALT) at baseline was 19.1%. There were 381 (38.3%) workers with long-term exposures to day-night rotating shift work (RSW). 14.2% of subjects developed MetS during follow-up. After 5 years, the workers with e-ALT had significantly unfavorable changes in MetS-components, and higher rates of MetS development, *vs* subjects with normal baseline ALT levels. Workers with both baseline e-ALT and 5-year persistent RSW (pRSW) exposure had the highest rate of MetS development. Also, e-ALT-plus-pRSW workers had a significant increase in MetS-components at

follow-up, compared with the other subgroups. After controlling for potential confounders, e-ALT-plus-pRSW workers posed a significant risk for MetS development (odds ratio, 2.7; 95% confidence interval, 1.4-5.3, *vs* workers without baseline e-ALT nor pRSW).

CONCLUSION: We suggest that all early middle-aged male employees with e-ALT should be evaluated and managed for MetS. Particularly in terms of job arrangements, impacts of long-term RSW on MetS development should be assessed for all male employees having baseline e-ALT.

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Key words: Alanine aminotransferase; Early middle aged; Male; Metabolic syndrome; Shift work

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INTRODUCTION

Elevated serum alanine aminotransferase (ALT) is a common abnormality of health examinations among the middle-aged working population^[1,2]. Nowadays, it is unavoidable that a large number of asymptomatic workers with elevated ALT (e-ALT), regardless of the underlying cause, are asked to do rotating shift work (RSW) on 24-h production lines^[3]. In previous studies, e-ALT^[4,5] and shift work^[6,7] had been independently assessed for their associations with metabolic syndrome (MetS), which has been linked with cardiovascular disease (CVD)^[8,9], one of the leading death causes among working populations^[10]. However, for early middle-aged workers with e-ALT in baseline conditions, few long-term follow-up studies assessed the association between RSW and MetS development. In terms of workplace health management and job arrangement, surveys for

the impact of RSW on MetS development among the subjects having e-ALT urgently required. In Taiwan, periodic health checkups are compulsorily for employees in many workplaces; thus we had the opportunity to utilize a retrospective follow-up study to assess the impact of RSW on MetS development among early-middle-aged workers having baseline e-ALT.

MATERIALS AND METHODS

Participants

In accordance with the Labor Health Protection Regulation of the Labor Safety and Health Act, 1203 male workers of an electronic manufacturing company underwent compulsory health checkups in 2002 and 2007. Final analysis of this follow-up study only included the subjects not fulfilling MetS criteria in 2002. Among the 1203 workers undergoing health examinations in both 2002 and 2007, 207 employees were excluded from study because they were screened previously with MetS. Final records of a total of 996 male workers constituted the cohort for the study and endpoint analysis. Hepatic virus infection is highly prevalent among Taiwanese adults^[2], our analysis included the hepatic virus carriers without MetS, and we controlled these factors in our final multivariate analysis. Most of the male employees of this electronics manufacturing company were residents of northern Taiwan.

The procedures and measurements of the health examinations took place in the morning, following an overnight fast, at a health-care unit of the factory. This health examination was open to all registered employees during every working day, 07:30 am to 09:30 am, for a 1-mo span. We suggested that the health checkups for the rotating shift workers be performed on the 3rd to 6th day of their day-duty. All the employees were requested to avoid drastic physical exercises (i.e. long-distance running, heavy weight lifting training etc) during the 3 d before undergoing the health examination.

Subjects' identities were anonymous and unlinked to the data. This analytical study, limited within health checkups records, followed the ethical criteria for human research, and the study protocol (TYGH09702108) was reviewed and approved by the Ethics Committee of the Tao-Yuan General Hospital, Taiwan.

Demographics, job types, lifestyle data, and biological measurements

In 2002, a questionnaire about baseline personal history, including smoking habits, physical exercise, drinking, and dietary habits, was completed by the examinees. The day-night RSW was determined from the self-reported questionnaires in 2002 and 2007. The shifts on the 24 h production line were scheduled on a three-team/two-shift plan (6 d-shifts-three rest days-six night-shifts-three rest days, *etc.*). The day and night shifts started at 07:30 am and 19:30 pm, respectively.

Physical examination and blood tests were performed on all participants in both 2002 and 2007. The participants arrived at the health care unit in the morning, between

07:30 am and 09:30 am, after an overnight 8 h fast. The physical examination records included measurements of waist circumference, weight, height, and blood pressure. Waist circumferences were measured midway between the lowest rib and the superior border of the iliac crest. After being seated for 5 min, sitting blood pressure was measured with the dominant arm using digital automatic sphygmomanometers (model HEM 907, Omron, Japan), twice at a 5-min intervals; the mean of the reading was used for data analysis. After the physical examination, participants were placed in a reclined position, and venous blood (20 mL) from an antecubital vein of the arm was taken for subsequent tests. Blood specimens were centrifuged immediately thereafter, and shipped in the frozen state, using dry ice, to a central clinical laboratory in the Tao-Yuan General Hospital (certified by ISO 15189 and ISO 17025). Glucose [glucose oxidase method, intra- and inter-assay coefficient of variation (CV) < 5%], triglyceride (enzymatic method, intra- and inter-assay CV < 5%), high-density lipoprotein (HDL) (enzymatic method, intra- and inter-assay CV < 5%), ALT, aspartate aminotransferase (Japan Society of Clinical Chemistry method, intra- and inter-assay CV < 5%) assays were conducted by a Hitachi autoanalyzer model 7150 (Hitachi, Tokyo, Japan). hepatitis B virus (HBV) surface antigen, anti-HBV surface antibody and anti-HCV antibody were measured by the microparticle enzyme immunoassay using the AxSym System instrument (Abbott, Illinois, USA), with the intra-assay and inter-assay CV < 10%. Insulin was determined with an AxSym autoanalyzer (Abbott, Illinois, USA) radioimmunoassay, with the intra-assay and inter-assay CV < 10%.

Abdominal sonographic examinations

Institutional Review Board of Tao-Yuan General Hospital did not agree to perform liver biopsies on apparently healthy subjects. Sonographic diagnosis for fatty liver is widely accepted in many epidemiological surveys^[11]; therefore, we used this noninvasive method to diagnose fatty liver. In 2002, abdominal ultrasound examinations were performed using convex-type real-time electronic scanners (Toshiba SSA-340 with 3.75 MHz convex-type transducer) by three gastrointestinal specialists. These gastrointestinal specialists had 10-15 years of experience in abdominal ultrasound examinations, and were blind to the medical history as well as to the blood test results of the examinees.

Definitions

MetS: The pre-MetS status was defined as one or two^[12], and the MetS designation was made if three or more of the following five criteria were fulfilled: Central obesity (waist circumference > 90 cm based on Taiwanese criteria^[13]); Elevated blood pressure (defined as systolic blood pressure $\geq$ 130 mmHg or diastolic blood pressure $\geq$ 85 mmHg; Hyperglycemia, hypo-HDL cholesterolemia and hypertriglyceridemia (defined based on modified National Cholesterol Education Program Adult Treatment Panel III criteria^[14] as: fasting sugar $\geq$ 100 mg/dL, HDL < 40 mg/dL, and triglycerides $\geq$ 150 mg/dL). We specified

the subjects with MetS outcome as those who were without MetS in 2002 but with MetS in 2007.

e-ALT: e-ALT was defined as ALT > 40 U/mL, according to the standard reference limits used at Tao-Yuan General Hospital and other by studies^[15].

Insulin resistance: Insulin resistance was calculated by the equation of homeostasis model assessment, $HOMA_{IR} = \text{fasting insulin (U/mL)} \times \text{glucose (mmol/L)} / 22.5$, and defined as the top quartile of $HOMA_{IR}$ ^[16].

Persistent RSW (pRSW): According to the Labor Health Protection Regulation of the Labor Safety and Health Act, periodical health examination ought to be performed on all registered employees working more than 1 year. These examinations took place annually for workers in this company. In the questionnaire, workers answered “yes” to “my job is a rotating shifting work”, indicating that the workers had done a rotating job for at least 1 year. Generally, this working pattern continues except for reasons of family events or promotions in this kind of factory. Those who did RSW for at least 1 year in the beginning and at the end of our follow-up were likely to have continued doing the same types of jobs. For the reasons mentioned above, we arbitrarily defined in our analysis of job type as: “pRSW” exposure was defined when the answers to the question in 2002 and 2007 were both day-night rotating shift work. Work information obtained through a self-administered questionnaire has been generally accepted in previous investigations^[6,17].

Lifestyle factors: For defining “ever been a smoker”, the question started with “Do you smoke? (1 Never, 2 Currently smoke, 3 Did but quit now)” and was followed by questions about quantity. We defined “ever been a smoker” as saying “yes” to question 2 or 3 and consuming at least 10 cigarettes daily for over 1 year. For defining “used to having snacks”, the question started with “Are you used to having snacks before sleeping?” and “Are you used to having snacks between meals?” and followed with questions about quantity. We defined the habits of having snacks in the present study as more than three times per week. The standard portions of examples for snacks (fruit products, milk products, fried food, nuts, beans products, meat, and alternatives) were explained to examinees in questionnaires. We defined “having routine physical exercise” as answering “doing exercises more than three times every week”. For defining “habitual drinker”, the question started with “Do you consume alcohol as usual? (1 No, 2 Yes)” and was followed by questions about frequency and quantity. We defined “habitual drinker” in this study as saying “yes” to question 2 and consuming it at least once per week for over 1 year.

Sonographic fatty liver: The definition of ultrasonic fatty liver was based on a comparative assessment of image brightness relative to the kidneys, under identical criteria^[18].

Statistical analysis

Baseline characteristics and abnormal rates were compared between four subgroups divided by e-ALT and pRSW using ANOVA and Tukey's test for continual variables and χ^2 test for categorical variables. Modes and ranges represented the initial, the 5th year and the changes of MetS-component numbers, were also tested by ANOVA and Tukey's test. Baseline and the 5th year abnormality rates were compared using the χ^2 test. Multivariate logistic regression, adjusted for age, baseline insulin resistance status, MetS-components, pRSW exposure, exercise, smoking, drinking and diet behaviors, viral hepatitis infection, and fatty liver was used to estimate the adjusted odds ratios (OR) and 95% confidence intervals (CI) of risk factors for predicting MetS development. A two-sided P -value < 0.05 was considered statistically significant. SAS version 8.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. Excel 2000 (Microsoft®, USA) was used for the statistical figure.

RESULTS

General and follow-up characteristics of the study population

Job descriptions are shown in Table 1. According to the characteristics of automatic production lines in such factories, most employees do light manual labor jobs or sedentary work. Tables 2 and 3 present baseline data of the overall and the four subgroups divided by initial e-ALT and pRSW exposures. Our sample population had an initial mean age of 32.1 years (SD 5.9 years); with total prevalence rates of e-ALT and pRSW of 19.1% and 38.3%, respectively. There were 14.2% (141/996) subjects who developed MetS within 5 years. At baseline, there were significant differences in age among the four subgroups; the subgroups with pRSW exposures were younger than the other two subgroups without pRSW exposures (30.9 and 31.4 years *vs* 32.8 and 32.6 years, Table 2). Baseline measurements of MetS-components, liver enzymes and insulin were significantly unfavorable in the two subgroups with e-ALT (Table 2). As shown in Table 3, baseline abnormality rates of MetS-components, hepatic virus infections, insulin resistance, and fatty liver were higher in the two groups with baseline e-ALT than in the subgroups with normal baseline ALT, though the baseline prevalence rates of central obesity and hyperglycemia were not significantly different among the four groups. Having snacks before bedtime and smoking habits prevailed in the group of pRSW with normal ALT, whereas other lifestyle parameters were not significantly different among the four groups. The overwhelming majority of pRSW workers (92.4%) were on-site workers (Tables 1 and 3).

Changes of MetS-components and its numbers within follow up

Most of the 5th year and 5-year changes of abnormality rates for MetS-component were unfavorable in the two subgroups with baseline e-ALT, and particularly in the subgroup with e-ALT-plus-pRSW (Table 4). The

Table 1 Job contents for different job types/e-ALT of the workers in this investigation *n* (%)

Job title	Routine work activities	<i>n</i> = 996	e-ALT: no; pRSW: no (<i>n</i> = 496)	e-ALT: no; pRSW: yes (<i>n</i> = 310)	e-ALT: yes; pRSW: no (<i>n</i> = 119)	e-ALT: yes; pRSW: yes (<i>n</i> = 71)
Operator	On-site work	232 (23.3)	101	97	17	17
Technician	On-site work	422 (42.4)	158	178	40	46
Engineer	On-site work	159 (16.0)	131	2	24	2
Team leader	On-site work	85 (8.5)	33	29	18	5
Specialist	On-site work	22 (2.2)	16	1	5	0
Deputy manager	Management	42 (4.2)	31	1	10	0
General manager	Management	19 (1.9)	15	0	4	0
Director	Management	3 (0.3)	3	0	0	0
Guard	Security	8 (0.8)	6	1	1	0
Clerk	Office work	4 (0.4)	2	1	0	1

e-ALT: Elevated alanine aminotransferase; pRSW: Persistent rotating shift work.

Table 2 Baseline characteristics of subjects stratified by e-ALT¹ and pRSW status *n* (%)

Measurement (SD)	Total population (<i>n</i> = 996)	e-ALT: no; pRSW: no (<i>n</i> = 496)	e-ALT: no; pRSW: yes (<i>n</i> = 310)	e-ALT: yes; pRSW: no (<i>n</i> = 119)	e-ALT: yes; pRSW: yes (<i>n</i> = 71)	<i>P</i> value ²
Age (yr)	32.1 (5.9)	32.8 (6.0)	30.9 (5.8)	32.6 (5.3)	31.4 (5.7)	0.0001
Body mass index (kg/m ²)	23.4 (2.9)	23.2 (2.8)	22.7 (2.8)	25.0 (2.5)	25.0 (3.0)	< 0.0001
Waist (cm)	78.3 (7.6)	78.1 (7.4)	76.4 (7.4)	82.5 (6.3)	81.3 (8.1)	< 0.0001
Systolic blood pressure (mmHg)	119.2 (14.1)	118.2 (13.8)	118.2 (14.3)	123.0 (14.0)	123.5 (14.1)	0.0002
Diastolic blood pressure (mmHg)	72.9 (9.0)	72.3 (8.6)	72.3 (8.9)	75.4 (10.0)	75.7 (9.3)	0.0002
Fasting blood glucose (mg/dL)	94.5 (14.8)	95.2 (18.6)	92.9 (8.1)	95.1 (8.1)	94.5 (15.2)	0.1797
Triglycerides (mg/dL)	113.1 (86.3)	109.3 (97.8)	101.0 (55.6)	149.3 (96.0)	132.2 (74.7)	< 0.0001
HDL cholesterol (mg/dL)	48.6 (11.0)	49.3 (10.9)	49.6 (11.4)	45.2 (10.8)	44.9 (7.9)	< 0.0001
Alanine aminotransferase (U/L)	31.0 (29.4)	21.7 (7.7)	21.1 (8.4)	68.8 (42.6)	75.8 (54.3)	< 0.0001
Aspartate aminotransferase (U/L)	25.8 (17.9)	21.9 (5.1)	21.8 (5.5)	39.3 (23.3)	48.5 (47.8)	< 0.0001
Insulin (mg/dL)	8.5 (6.2)	8.0 (4.9)	7.4 (5.2)	11.4 (7.8)	11.6 (11.4)	< 0.0001

¹ALT > 40 mg/dL; ²ANOVA was conducted, using Tukey's test.Table 3 Baseline abnormality rates of subjects stratified by e-ALT¹ and pRSW status

Abnormalities	Total population (<i>n</i> = 996)	e-ALT: no; pRSW: no (<i>n</i> = 496)	e-ALT: no; pRSW: yes (<i>n</i> = 310)	e-ALT: yes; pRSW: no (<i>n</i> = 119)	e-ALT: yes; pRSW: yes (<i>n</i> = 71)	<i>P</i> value ²
MetS-component abnormality (%)						
Central obesity	3.6 (36/996)	2.8	3.2	5.9	7.0	0.1595
Hyperglycemia	20.7 (206/996)	22.0	18.4	22.7	18.3	0.5658
Hypertriglyceridemia	17.5 (174/996)	15.1	13.2	34.5	23.9	< 0.0001
Elevated blood pressure	21.3 (212/996)	18.3	20.6	29.4	31.0	0.01
Hypo-HDL cholesterol	20.2 (201/996)	18.5	17.1	32.8	23.9	0.0018
Elevated alanine aminotransferase	19.1 (190/996)					
Hepatovirus B carrier	19.3 (192/996)	18.1	14.8	27.7	32.4	0.0005
Hepatovirus C carrier	1.2 (12/996)	0.4	0.3	4.2	5.6	< 0.0001
Fatty Liver	30.8 (307/996)	24.0	21.0	66.4	62.0	< 0.0001
Lifestyle factor (%)						
Ever been a smoker (≥ 1 yr)	42.7 (425/996)	36.9	51.6	39.5	49.3	0.0003
Having snacks before sleeping (≥ 1 d/wk)	42.5 (423/996)	37.7	51.9	39.5	39.4	0.0008
Having snacks between meals (≥ 1 d/wk)	40.9 (407/996)	40.5	39.4	47.1	39.4	0.5192
Physical exercise (≥ 3 times/wk)	34.6 (345/996)	38.5	31.6	31.9	25.4	0.0545
Habitual drinker (≥ 1 d/wk)	10 (99/996)	8.7	10.6	10.1	15.5	0.3196
Workplace factor (%)						
Persistent day-night rotating shift work	38.3 (381/996)					
On-site worker	92.4 (920/996)	88.5	99.1	87.4	98.6	< 0.0001
Development of MetS within 5 yr	14.2 (141/996)	11.1	8.7	27.7	36.6	< 0.0001

¹ALT > 40 mg/dL; ²χ² test was conducted for abnormalities and four liver function/shift work subgroups. MetS: Metabolic syndrome.

male workers having both baseline e-ALT and 5-year exposures to pRSW had the highest increasing rates of hyperglycemia (15.5% increased), hypertriglyceridemia

(21.1% increased) and elevated blood pressure (31.0% increased). They also displayed the highest rate of MetS development (36.6%) among four subgroups. As

Table 4 Change of abnormalities based on a 5-year follow-up among the subgroups stratified by ALT and shift work (%)

MetS and its components	e-ALT initially	pRSW exposure	Baseline	P value ¹	5th yr	P value ¹	Δ 5th yr vs baseline
Central obesity	No	No	2.8	0.1595	22.2	0.0037	19.4
		Yes	3.2		20.0		16.8
	Yes	No	5.9		33.6		27.7
		Yes	7.0		33.8		26.8
Hyperglycemia	No	No	22.0	0.5658	16.7	< 0.0001	-5.2
		Yes	18.4		13.9		-4.5
	Yes	No	22.7		26.9		4.2
		Yes	18.3		33.8		15.5
Hypertriglyceridemia	No	No	15.1	< 0.0001	26.8	< 0.0001	11.7
		Yes	13.2		25.5		12.3
	Yes	No	34.5		42.0		7.6
		Yes	23.9		45.1		21.1
Elevated blood pressure	No	No	18.3	0.01	48.0	0.1025	29.6
		Yes	20.6		50.0		29.4
	Yes	No	29.4		55.5		26.1
		Yes	31.0		62.0		31.0
Hypo-HDL cholesterol	No	No	18.5	0.0018	6.3	< 0.0001	-12.3
		Yes	17.1		8.1		-9.0
	Yes	No	32.8		17.6		-15.1
		Yes	23.9		23.9		0.0
Development of MetS within 5 yr	No	No			11.1	< 0.0001	
		Yes			8.7		
	Yes	No			27.7		
		Yes			36.6		
MetS-component numbers (mode)	No	No	0		1		0
		Yes	0		1		0
	Yes	No	2		1		0
		Yes	1		2		1

¹ χ^2 test was conducted, four abnormalities of subgroups were constructed. Δ: Differences between 2007 and 2002; plus indicates increasing and minus indicates decreasing trend within follow-up.

Table 5 Multivariate analysis for the risk of development of metabolic syndrome among male workers

Adjustment for initial characteristics			OR ¹ (e-ALT: no vs pRSW: no)	95% CI
Model 1	Age (yr)	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	3.1	1.9-5.0
		e-ALT: yes; pRSW: yes	4.7	2.7-8.2
Model 2	Age, MetS-components, insulin resistance	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.8	1.03-3.0
		e-ALT: yes; pRSW: yes	3.8	2.0-6.9
Model 3	Age, MetS-components, insulin resistance, hepatovirus infections	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.9	1.1-3.2
		e-ALT: yes; pRSW: yes	4.0	2.1-7.4
Model 4	Age, MetS-components, insulin resistance, hepatovirus infections, fatty liver	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.4	0.8-2.4
		e-ALT: yes; pRSW: yes	2.9	1.5-5.5
Model 5	Age, MetS-components, insulin resistance, hepatovirus infections, fatty liver, lifestyle and workplace factors	e-ALT: no; pRSW: yes	0.7	0.4-1.2
		e-ALT: yes; pRSW: no	1.3	0.7-2.3
		e-ALT: yes; pRSW: yes	2.7	1.4-5.3

¹Each variant put into the model as categorical data. OR: Odds ratio; CI: Confidence interval.

shown on figure, the subgroup of e-ALT-plus-pRSW had a significantly increasing shift in numbers of MetS-component.

Risk assessment for MetS development

Table 5 shows the age-adjusted OR for MetS obtained by the step-by-step examinations. At the beginning, the age-adjusted OR of MetS development for the e-ALT workers without or with pRSW exposure

were 3.1 (95% CI: 1.9-5.0) and 4.7 (95% CI: 2.7-8.2) respectively. Combined with step by step examinations, after controlling for confounders of initial age, MetS-components, insulin resistance, liver status, lifestyle and workplace factors, the multivariate analysis indicated that the male workers with baseline e-ALT plus pRSW remained at a 2.7-fold (95% CI: 1.4-5.3) greater risk of developing MetS compared with those having neither initial e-ALT nor pRSW exposure. Male employees who

had initial e-ALT but without pRSW had an increased risk for progression to MetS when compared with those who initially had normal ALT. This result was significant but became insignificant when fatty liver was introduced as one of the controlling factors: adjusted OR of MetS development became to 1.3 (95% CI: 0.7-2.3) for the workers having initial e-ALT but without pRSW (Table 5).

DISCUSSION

All the subjects in our sample population continued working in the company at least for 5 years, which reveals that our workers were in relatively stable occupational situations. Our conclusions from observations of a large and stable middle-aged working population will contribute to the surveys on MetS in such working groups.

e-ALT and MetS development

The baseline e-ALT prevalence rate (19.1%) in our sample population was as high as that in other working populations^[1]. Although not fulfilling MetS criteria at screening, the workers with e-ALT had significantly more unfavorable baseline measurements, as well as higher abnormality rates for most of the MetS-components (Tables 2 and 3), as compared with workers having normal baseline ALT. Similar findings were revealed in a previous cross-sectional study for young healthy men^[19]. Our follow-up results (Table 4) demonstrated that the subjects initially having e-ALT tended display considerably raised abnormality rates of MetS-components within 5 years. Moreover, at the end of follow-up, the two subgroups with baseline e-ALT had significantly higher rates of MetS development than the other two subgroups with normal baseline ALT levels.

As a mortality predictor^[20], e-ALT represents many detrimental health conditions, including systemic inflammation^[19,20], which was reported to contribute to MetS development. In our step-by-step multivariate analysis (Table 5), the significant impacts of baseline e-ALT on MetS development among the workers without pRSW remained until we introduced an operator-dependent survey, fatty liver^[21], which has an extremely close relationship with e-ALT^[15], as a controlling factor. According to our statistical analysis, baseline e-ALT is associated with long-term development of manifold metabolic abnormalities and MetS among early middle-aged male workers. We suggest workplace MetS managements for all workers having e-ALT.

Changes of MetS-components among our male workers within 5 years

Taking each MetS-component into consideration, most of the MetS-components abnormality rates increased as our population got older (Table 4); though some exceptions for hyperglycemia and hypo-HDL cholesterolemia were found in the subgroups with normal baseline ALT or without pRSW exposure.

We demonstrated that the early middle-aged male workers with baseline e-ALT had increased abnormality rates of hyperglycemia, same as previous findings^[22].

Similarly, a significantly increased prevalence of hyperglycemia was found for workers with e-ALT-plus-pRSW, which was compatible with the findings that shift work can aggravate the insulin resistance^[23]. In contrast, for the workers with normal baseline ALT levels, hyperglycemia showed an improving trend. These phenomena of improved blood sugar were dissimilar to the elderly population^[24], but have been shown in many follow-up observations for healthy adults^[22,25]. Our early middle-age subjects with normal baseline ALT seemed to maintain the ability to stabilize blood sugar. In contrast, the subjects with baseline e-ALT displayed impaired abilities to improve their blood sugar levels, which might be related to pRSW exposure. Also, hypo-HDL was associated with an improving trend among our early-middle-aged males during follow-up, except for the e-ALT-plus-pRSW workers. This increasing trend of HDL has been mentioned among healthy adults^[26], but not found in the elderly^[24]. As apparently healthy young adults get older, HDL might quantitatively increase in response to external stimuli^[24,27] or internal challenges^[28], and this increase is likely to reach a plateau^[29]. Our comparatively young healthy workers might not yet have reached their plateau, so they might have increasing HDL concentrations within our follow-up. However, when under chronic oxidative stress caused by simultaneous e-ALT^[30] and shift work exposure^[31], our early middle-aged subjects might have significantly weaker capabilities for quantitatively increasing HDL.

Increasing MetS-component numbers and development of MetS among the workers with both baseline e-ALT and pRSW exposures

Analyzing the number of MetS-components is informative in determining the CVD risk of the general population^[9]. In addition, MetS-component numbers were reported in many convincing studies that closely linked them with damaged conditions of the whole human body: impaired cardiovascular conditions^[32,33], cognitive impairment^[34], and colon polyps^[35]. Shift work is associated with the premature aging process^[36], and as demonstrated in our analyses (Table 4 and Figure 1), the e-ALT-plus-pRSW workers had significantly increased numbers of MetS-components in the end of follow-up. Thus, the impacts of shift work exposure on general health conditions of the e-ALT workers should receive close attention.

Some factors might confound our findings, such as: high prevalence of HBV and HCV among those with e-ALT at baseline, fatty liver might be linked with HCV carrier status, and the reduction in the amount of exercise in individuals who developed MetS. However, in the analysis carried out in consecutive steps for the male workers with e-ALT, all the other confounders did not remove the significance of pRSW on MetS development (Table 5, Model 3, 4 and 5). Our follow-up observations confirmed that persistent day-night RSW accelerates the progression toward MetS among the e-ALT workers. e-ALT can work with other risk factors to worsen the inflammatory state^[30], and shift work brings about long-term oxidative stress^[31], therefore a possible explanation for our male shift workers with e-ALT tending to develop

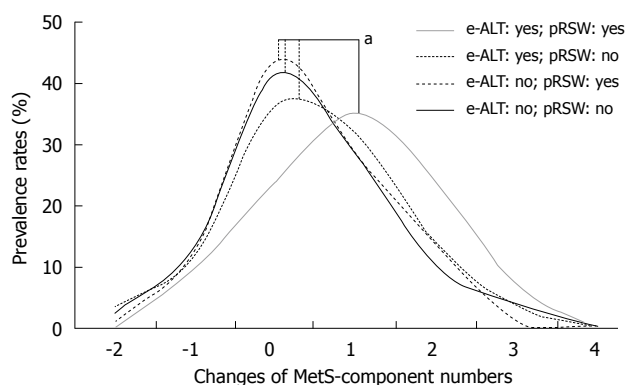


Figure 1 Distributions of MetS-components number changes at 5-year follow-up, sub-grouped by elevated alanine aminotransferase and persistent rotating shift work. ^aANOVA was conducted, using Tukey's test; $P = 0.0015$. e-ALT: Elevated alanine aminotransferase; pRSW: Persistent day-night rotating shift work; MetS: Metabolic syndrome.

MetS might be aggravated systemic chronic inflammatory reactions^[37,38].

Due to the dramatically rising occurrence rate of MetS, we strongly suggest screening, follow-up, and treatment of MetS for workers with both e-ALT and unavoidable long-term rotational shift work exposures.

Potential limitations

Some potential limitations of our analysis need to be considered.

Firstly, Taiwan National Health Insurance has provided comprehensive medical care, and for the pre-MetS subjects, it was possible that they could have had corrective management during our follow-up, and those actions might have led to protective effects^[39]. In such a case, the main limitations of this observational investigation would be that we did not take into consideration the potential treatment effects for the at risk workers. Thus, our conclusion might be affected by the results of therapeutic management programs, and we might be underestimating risk factors for MetS development. Secondly, our shift workers with normal ALT were relatively young in our sample population (Table 2), as compared with workplace populations of other studies^[6]. Physically, the advantage of youthfulness^[6] might lead us to a statistically insignificant conclusion for the shift work exposures on MetS development among subjects with normal ALT (Table 5); the aging effect on MetS development is an important issue for such a young cohort, and requires further surveys. Lastly, we proposed that MetS development among our e-ALT shift workers might be closely linked to chronic inflammation; therefore direct inflammatory evidence^[19] should be carefully surveyed in future investigations.

As for the increasing body of professional women occupying workplaces, it is important to compare the differences between genders in risk assessments for MetS development; thus all the present findings are worthy of being tested in future studies of female workers. In terms of health and safety, for the individuals who initially had MetS and were excluded of this study, it is necessary to assess their cardiovascular outcomes in future investigations.

In conclusion, for early middle-aged male workers, baseline and changes of MetS component abnormalities, as well as the development of MetS, are all associated with baseline e-ALT. In addition, long-term RSW exposures significantly aggravate MetS development among the workers having baseline e-ALT. We suggest all male workers having e-ALT should be carefully evaluated and managed for MetS. Particularly in terms of job arrangements, impacts of long-term RSW on MetS development should be assessed for all male employees having baseline e-ALT.

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COMMENTS

Background

Elevated serum alanine aminotransferase (ALT) is a common abnormality of health examinations among middle-aged working populations. It is unavoidable nowadays that a large number of asymptomatic workers with elevated serum ALT levels might be asked to do rotating shift work (RSW) on 24-h production lines. In terms of workplace health management and job assignment, a 5-year follow-up study assessing the association between RSW and metabolic syndrome (MetS) development was conducted in Taiwan for male workers.

Research frontiers

In some studies, elevated ALT (e-ALT) and shift work had been independently assessed for their associations with MetS, which is associated with cardiovascular disease, one of the leading causes of death among working populations. This survey takes these two risk factors together into consideration and obtained significant findings.

Innovations and breakthroughs

Workers who had both baseline e-ALT and persistent RSW (pRSW) exposures (e-ALT-plus-pRSW workers) had a significantly high risk for MetS development among middle-aged male workers. In addition, e-ALT-plus-pRSW workers had a significant increase in MetS-component numbers at the end of follow-up, compared with the other workers. Finally, e-ALT-plus-pRSW workers had a significant risk for MetS development.

Applications

All the workers with e-ALT should be carefully evaluated and managed for MetS. Particularly, MetS risk assessment must be emphasized for male employees having e-ALT and facing long-term RSW exposures.

Peer review

The public health expert reviewers agreed the important area of research given the amount of shift work performed around the globe; they also pointed out that, given the large number of individual records and individuals examined, this is an important 5 years retrospective analysis of associated factors.

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ORIGINAL ARTICLE

Intraluminal *versus* infiltrating gallbladder carcinoma: Clinical presentation, ultrasound and computed tomography

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Abstract

AIM: To compare clinical presentation and ultrasound (US) and computed tomography (CT) sensitivity between intraluminal and infiltrating gallbladder carcinoma (GBCA).

METHODS: This retrospective study evaluated 65 cases of GBCA that were categorized morphologically into the intraluminal-GBCA ($n = 37$) and infiltrating-GBCA

($n = 28$) groups. The clinical and laboratory findings, presence of gallstones, gallbladder size, T-staging, nodal status, sensitivity of preoperative US and CT studies, and outcome were compared between the two groups.

RESULTS: There were no significant differences between the two groups with respect to female predominance, presence of abdominal pain, serum aminotransferases level, T2-T4 staging, and regional metastatic nodes. Compared with the patients with intraluminal-GBCA, those with infiltrating-GBCA were significantly older (65.49 ± 1.51 years *vs* 73.07 ± 1.90 years), had a higher frequency of jaundice (3/37 patients *vs* 13/28 patients) and fever (3/37 patients *vs* 10/28 patients), higher alkaline phosphatase (119.36 ± 87.80 IU/L *vs* 220.68 ± 164.84 IU/L) and total bilirubin (1.74 ± 2.87 mg/L *vs* 3.50 ± 3.51 mg/L) levels, higher frequency of gallstones (12/37 patients *vs* 22/28 patients), smaller gallbladder size (length, 7.47 ± 1.70 cm *vs* 6.47 ± 1.83 cm; width, 4.21 ± 1.43 cm *vs* 2.67 ± 0.93 cm), and greater proportion of patients with < 12 mo survival (16/37 patients *vs* 18/28 patients). The sensitivity for diagnosing intraluminal-GBCA with and without gallstones was 63.6% and 91.3% by US, and 80% and 100% by CT, respectively. The sensitivity for diagnosing infiltrating-GBCA with and without gallstones was 12.5% and 25% by US, and 71.4% and 75% by CT, respectively.

CONCLUSION: In elderly women exhibiting small gallbladder and gallstones on US, especially those with jaundice, fever, high alkaline phosphatase and bilirubin levels, CT may reveal concurrent infiltrating-GBCA.

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Key words: Gallbladder neoplasms; Carcinoma; Ultrasound; Computed tomography

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INTRODUCTION

Gallbladder carcinoma (GBCA) is the sixth most common gastrointestinal malignancy in the United States, following cancer of the colon, pancreas, stomach, liver, and esophagus^[1,2]. Nevertheless, GBCA is relatively uncommon with an estimated annual incidence of 1 or 2 cases per 100 000 people^[2]. The nonspecific nebulous symptoms associated with GBCA make the early diagnosis of this uncommon entity a challenge. Approximately 50% of cases of GBCA are found incidentally during cholecystectomy or surgery for other reasons^[3,4]. GBCA is a highly aggressive neoplasm with a 5-year survival rate of only 5%^[1-5]. Although most patients present with advanced disease, aggressive radical surgical resection of GBCA has been reported with encouraging results^[3-5]. Furthermore, survival of GBCA can be improved if early detection of the tumor can be achieved so that curative resection can be performed. Ultrasound (US) is the main initial diagnostic tool for suspected biliary lesions. It may be helpful for detecting GBCA although the infiltrative morphology of some tumors and the presence of gallstones, inflammation and debris may preclude tumor detection^[6-9]. Computed tomography (CT) has been reported as a comprehensive tool for imaging and staging of GBCA^[7-11].

To the best of our knowledge, the influence of different tumor morphology on clinical presentation, as well as on US and CT sensitivity of GBCA detection, have not been well studied. The purpose of this study was to analyze the differences in clinical presentation and sensitivity of preoperative US and CT assessment between GBCA presenting as an intraluminal mass and an infiltrating tumor.

MATERIALS AND METHODS

Patients

The computer data bank of our hospital was searched for cases from 1995 to 2008 with the index terms "malignant neoplasm of gallbladder and extrahepatic bile ducts" and "malignant neoplasm of gallbladder" (International Classification of Diseases, 9th Revision, clinical modification code = 156, 156.0). The medical records of all patients with these discharge diagnoses were reviewed. Patients who had surgery and pathologically proven GBCA, as well as preoperative US and/or CT were included in this case series. The patients were categorized into two groups according to the gross tumor morphology and histopathological features of the GBCA. The intraluminal-GBCA group comprised patients with intraluminal tumor growths that protruded into the gallbladder lumen, which grossly appeared as polypoid, fungating or large intraluminal masses. The infiltrating-GBCA group consisted of patients with infiltrating carcinomas that appeared grossly as focal or diffuse areas of wall thickening, or induration in the gallbladder wall. This retrospective study was approved by the institutional review board of our hospital and informed consent was waived due to the retrospective and anonymous nature of the analysis.

Imaging evaluation

Transabdominal US was performed with various scan-

ners including the Aloka SSD-650 (Aloka Co. Ltd., Tokyo, Japan), and the Acuson 128 XP/10 and Sequoia 512 scanners (Acuson, Mountain View, CA, USA) using a 3.5-MHz sector transducer. Abdominal CT was performed using a ProSpeed scanner (GE Healthcare, Milwaukee, WI, USA) or a Somatom Plus 4 scanner (Volume Zoom; Siemens, Forchheim, Germany) from the liver dome to the pelvic floor. An intravenous bolus of 85-100 mL iodinated contrast material (60%-76% diatrizoate meglumine) was injected at a rate of 2-3 mL/s *via* the antecubital vein before scanning. All images were reconstructed at intervals of 5-10 mm. Starting in 2003, all CT images were interpreted on a high resolution monitor (MGD 521MK II; BarcoView, Kortrijk, Belgium) *via* the picture archiving and communication systems (PACS) system (Centricity Workstation, version 2.0; GE Healthcare). All CT images recorded in hard copy before 2003 were scanned into the PACS system for review, and all measurements were done by applying the tools provided by the Centricity Workstation. A detailed retrospective review of the medical records, US and CT images and pathological findings of each patient was conducted jointly by two radiologists, a surgeon and a pathologist, and any discordance was resolved by consensus.

Measurements

The clinical features (age, sex and presenting symptoms and signs), laboratory data (serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and total bilirubin levels), and pathological findings (gallstones, tumor morphology, gallbladder size, histopathological evaluation of T-staging and nodal metastasis) were recorded. With pathology as the gold standard, the true-positive and false-negative results of preoperative US and CT were obtained, and the sensitivity of the imaging modalities was determined. The outcomes of our patients were also documented.

Statistical analysis

The data are presented as mean $\pm$ SD or number (%). The clinical, laboratory and pathological findings were analyzed and compared between the intraluminal-GBCA and infiltrating-GBCA group using the Wilcoxon rank sum test for continuous variables and Fisher's exact test or the χ^2 test for categorical variables. The sensitivity of preoperative US and CT for the diagnosis of GBCA between the two groups with and without gallstones also was analyzed. Statistical analysis was performed with SYSTAT software (SPSS, version 11.0, Chicago, IL, USA) and $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and laboratory information

Eight of 73 patients with a discharge diagnosis of GBCA were excluded from further study. Three of these patients refused surgery; three were considered poor candidates for surgery because of concurrent cardiac or pulmonary disease or old age; and the other two lacked preoperative US or CT images for review. A total of 65 patients who fulfilled the inclusion criteria were included. According to

the pathological findings, 37 patients (female:male = 23:14; mean age, 65.5 years) formed the intraluminal-GBCA group and the other 28 patients (female:male = 18:10; mean age, 73.1 years) formed the infiltrating-GBCA group.

The clinical and pathological data of both groups are compared in Table 1. Female preponderance was noted in both groups but the patients in the infiltrating-GBCA group were significantly older than those in the intraluminal-GBCA group ($P = 0.02$). The presenting symptoms were, in combination or alone, right upper abdominal or epigastric pain or discomfort in 51 patients, jaundice in 23 and fever in 13. Ten patients were asymptomatic and were diagnosed incidentally. The infiltrating-GBCA group had a higher frequency of jaundice (46.4% *vs* 8.1%, $P < 0.001$) and fever (35.7% *vs* 8.1%, $P = 0.006$) than the intraluminal-GBCA group. There were no significant differences in the serum aspartate and alanine aminotransferase levels between the groups. However, the infiltrating-GBCA group had significantly higher levels of serum alkaline phosphatase (220.68 IU/L *vs* 119.36 IU/L, $P = 0.03$) and total bilirubin (3.50 mg/dL *vs* 1.74 mg/dL, $P = 0.011$) than the intraluminal-GBCA group.

Pathological findings

The histological types in the intraluminal-GBCA group included 34 adenocarcinomas, one papillary adenocarcinoma, one sarcomatoid adenocarcinoma and one adenosquamous carcinoma. The maximal tumor length ranged from 1.1 to 3.8 cm (mean 2.3 cm). Among 34 intraluminal adenocarcinomas, five patients had no associated gallstones, while histopathological examinations revealed tumor development from the tubular or villotubular polypoid adenomas. The histological types in the infiltrating-GBCA group included 24 adenocarcinomas, two adenosquamous carcinomas, one clear cell carcinoma and one squamous cell carcinoma. The maximal thickness of the tumor ranged from 0.7 to 1.2 cm (mean 0.9 cm). Thirty-four of these 65 patients had gallstones and the number of stones ranged from one to 35. Twenty-six of these patients had > 3 stones with sizes ranging from 0.5 to 2.9 cm, and the other eight patients had 1-3 stones, with sizes varying from 0.3 to 2.7 cm. The infiltrating-GBCA group exhibited a significantly higher frequency of gallstones than the intraluminal group (78.6% *vs* 32.4%, $P < 0.001$). Compared with the intraluminal-GBCA group, the infiltrating-GBCA group exhibited a significantly shorter gallbladder length (7.47 cm *vs* 6.47 cm, $P = 0.032$) and width (4.21 cm *vs* 2.67 cm, $P = 0.026$), and had a significantly lower frequency of T1 tumors (21.7% *vs* 3.6%, $P = 0.038$). On the other hand, there were no significant differences between our two groups with respect to the frequencies of T2, T3, or T4 tumors, and the presence of regional metastatic lymph nodes.

Preoperative US and CT

Comparison of preoperative US and CT diagnosis is summarized in Table 2. Twelve out of 37 (32.4%) patients in the intraluminal mass group had gallstones and 11 of them underwent US, with a sensitivity of 63.6% for preoperative diagnosis of GBCA, while five underwent CT, with a sensitivity of 80%. Twenty-two out of 28

Table 1 Clinical and pathological data from 37 patients with GBCA presented as intraluminal mass, and 28 patients with GBCA presented as infiltrating tumor (mean $\pm$ SD) n (%)

	Intraluminal mass	Infiltrating tumor	<i>P</i> value
Age (yr) ²	65.49 $\pm$ 1.51	73.07 $\pm$ 1.90	0.020 ^a
Sex ratio (female) ¹	23 (62.1)	18 (64.3)	0.861
RUQ or epigastric pain or discomfort ¹	28 (75.7)	23 (82.1)	0.530
Jaundice ¹	3 (8.1)	13 (46.4)	$< 0.001^a$
Fever ¹	3 (8.1)	10 (35.7)	0.006 ^a
Incidental finding	7 (18.9)	3 (8.1)	0.364
Aspartate aminotransferase (IU/L) ²	89.36 $\pm$ 101.14	74.37 $\pm$ 88.62	0.914
Alanine aminotransferase (IU/L) ²	113.21 $\pm$ 103.97	78.87 $\pm$ 82.04	0.252
Alkaline phosphatase (IU/L) ²	119.36 $\pm$ 87.80	220.68 $\pm$ 164.84	0.030 ^a
Total bilirubin (mg/dL) ²	1.74 $\pm$ 2.87	3.50 $\pm$ 3.51	0.011 ^a
Presence of gallstones ¹	12 (32.4)	22 (78.6)	$< 0.001^a$
Gallbladder size			
Length (cm) ²	7.47 $\pm$ 1.70	6.47 $\pm$ 1.83	0.032 ^a
Width (cm) ²	4.21 $\pm$ 1.43	2.67 $\pm$ 0.93	0.026 ^a
T1 tumor ¹	8 (21.7)	1 (3.6)	0.038 ^a
T2 tumor ¹	11 (29.7)	9 (32.1)	0.835
T3 tumor ¹	17 (45.9)	16 (57.1)	0.267
T4 tumor ¹	1 (2.7)	2 (7.2)	0.581
Metastatic lymph nodes ¹	13 (35.1)	15 (53.6)	0.712
Survival < 12 mo ¹	16 (43)	18 (68)	0.017 ^a

¹Fisher's exact test or χ^2 test; ²Wilcoxon rank sum test; ^aSignificant at 0.05; RUQ: Right upper quadrant; GBCA: Gallbladder carcinoma.

(78.6%) patients in the infiltrating-GBCA group had gallstones and 16 of them underwent US, with a sensitivity of only 12.5% for preoperative diagnosis of GBCA, while 14 underwent CT, with a sensitivity of 71.4%.

Twenty-five out of 37 (67.64%) patients in the intraluminal mass group had no gallstones and 23 of them underwent US, with a sensitivity of 91.5% for preoperative diagnosis of GBCA, while 20 underwent CT, with a sensitivity of 100%. Only six out of 28 (21.4%) patients in the infiltrating-GBCA group had no gallstones and four of them underwent US, with a sensitivity of only 25% for preoperative diagnosis of GBCA, while four underwent CT, with a sensitivity of 75%.

None of the 22 patients (15 without and seven with gallstones) in the intraluminal-GBCA group with both preoperative US and CT (Figures 1 and 2), had a false-negative result for GBCA. However, among 10 patients (two without and eight with gallstones) in the infiltrating-GBCA group, US and CT results were true-positive for GBCA in only four patients (Figure 3). The US results were false-negative for GBCA in the remaining six patients (five with gallstones), and CT was true-positive in four (Figures 4 and 5).

Outcomes

In the intraluminal-GBCA group, T1 tumors were found in eight patients, T2 in 11, T3 in 17, and T4 in one. Follow-up ranged from 3 to 115 mo (average 28.8 mo) and 16 patients (43%) survived < 12 mo (average survival, 9 mo). In the infiltrating-GBCA group, a T1 tumor was found in only one patient, T2 in nine, T3 in 16, and T4

Table 2 Comparison of preoperative US and CT diagnosis of 37 patients with GBCA presented as intraluminal mass, and 28 patients with GBCA presented as infiltrating tumor

	Intraluminal mass	Infiltrating tumor
With gallstones	12	22
Preoperative US	11	16
True positive	7	2
False negative	4	14
Sensitivity (%)	63.6	12.5
Preoperative CT	5	14
True positive	4	10
False negative	1	4
Sensitivity (%)	80.0	71.4
Without gallstones	25	6
Preoperative US	23	4
True positive	21	1
False negative	2	3
Sensitivity (%)	91.3	25.0
Preoperative CT	20	4
True positive	20	3
False negative	0	1
Sensitivity (%)	100	75

US: Ultrasound; CT: Computed tomography.

in two. Follow-up ranged from 1 to 160 mo (average 15.5 mo) and 19 patients (68%) survived < 12 mo (average survival, 4 mo). The percentage of patients with < 1-year survival in the infiltrating group was significantly higher than in the intraluminal-GBCA group ($P = 0.017$).

DISCUSSION

GBCA is a relatively uncommon cancer and is found incidentally in < 1% of 750 000 open cholecystectomies performed annually for gallstones in the United States^[12]. Although GBCA is characterized by early local invasion of the liver and biliary tree, early diagnosis remains difficult because most patients present with nonspecific abdominal symptoms^[2-5]. In the present study, most patients in both groups harbored T2 and T3 tumors and complained of chronic right upper quadrant or epigastric discomfort at presentation, but our results showed that intraluminal and infiltrating-GBCA might demonstrate different clinical manifestations. In patients with intraluminal-GBCA, T1 tumors were detected more commonly by US or CT incidentally as an asymptomatic gallbladder nodule, while patients with infiltrating GBCA exhibited a higher frequency of jaundice and fever, which mimicked acute cholecystitis. Although both groups showed similar abnormal mean levels of serum aminotransferases, patients with infiltrating-GBCA had significantly higher levels of serum alkaline phosphatase (220.7 IU/L *vs* 119.4 IU/L) and total bilirubin (3.5 mg/dL *vs* 1.7 mg/dL) than patients with intraluminal-GBCA.

Epidemiological studies have shown that female sex, age, postmenopausal status, ethnic differences and gallstones are the most well-known factors for GBCA development^[9,13-16]. A high prevalence of GBCA has been reported in New Mexico, Bolivia, Chile, Israel and Northern Japan, and a high prevalence of gallstones in these ethnic groups has also been found^[14,15]. Chronic

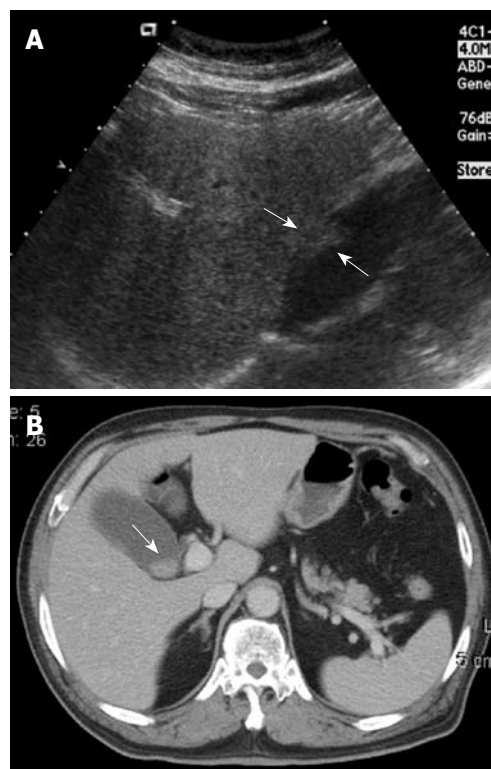


Figure 1 A 68-year-old woman with intermittent dull right upper quadrant pain for 6 mo. A: Ultrasound (US) showed a sessile polypoid lesion (arrows) with a mildly uneven surface; B: Contrast-enhanced computed tomography (CT) showed an intraluminal polypoid lesion (arrow) with no apparent enlarged regional lymph node.

irritation of the gallbladder caused by gallstones and tumor-promoter activity in the bile in cholelithiasis patients may account for the development of GBCA^[16,17]. In the present study, gallstones were identified in 34/67 patients (54%), with a lower incidence than in previous reports (64%-98%)^[18-20]. Of note, our results showed that significantly more patients with infiltrating-GBCA (22/28 patients, 78.6%) harbored coexisting gallstones than patients with intraluminal-GBCA (12/37 patients, 32.6%). Coupled with the fact that patients with infiltrating-GBCA were significantly older (mean age, 73 years) and had significantly smaller gallbladders than those with intraluminal-GBCA, we concur with the postulation that gallstones with a long duration of gallbladder wall irritation may lead to mucosal dysplasia and subsequent neoplasia^[19].

In contrast, more than two-thirds of patients with intraluminal-GBCA had no gallstones, and other predisposing factors of GBCA development including polypoid gallbladder lesions, choledochal cyst, pancreatobiliary duct anomalies, sclerosing cholangitis, porcelain gallbladder, cigarette smoking, typhoid carrier state, certain occupational and environmental carcinogens, and hormonal changes in women have also been described^[9,13-15]. None of our patients had any history of congenital bile duct or pancreatobiliary lesions, porcelain gallbladder or sclerosing cholangitis, but they did have other less common factors such as chemical or carcinogen exposure. Gallbladder polyps that are > 1 cm, single,

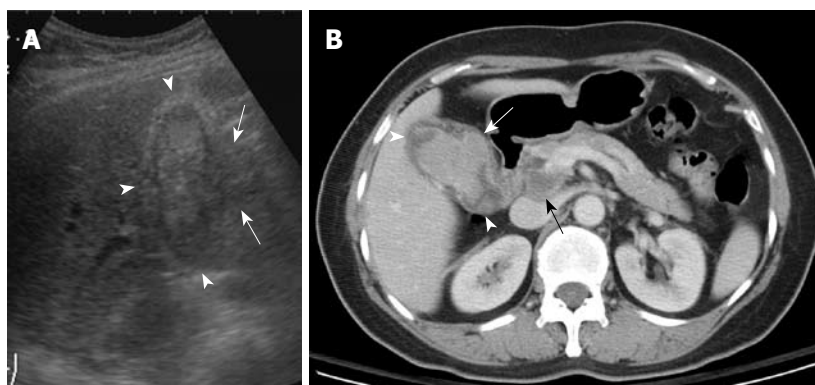


Figure 2 A 78-year-old woman with right upper abdominal pain and progressive jaundice for 2 mo. A: US showed an intraluminal heteroechoic mass that occupied nearly the whole gallbladder (arrowheads), with focal extraluminal invasion (arrows); B: Contrast-enhanced CT showed a large lobulated mass within the gallbladder (arrowheads), with extracholecystic invasion (white arrow) and hepatoduodenal ligament lymph node metastasis (black arrow).

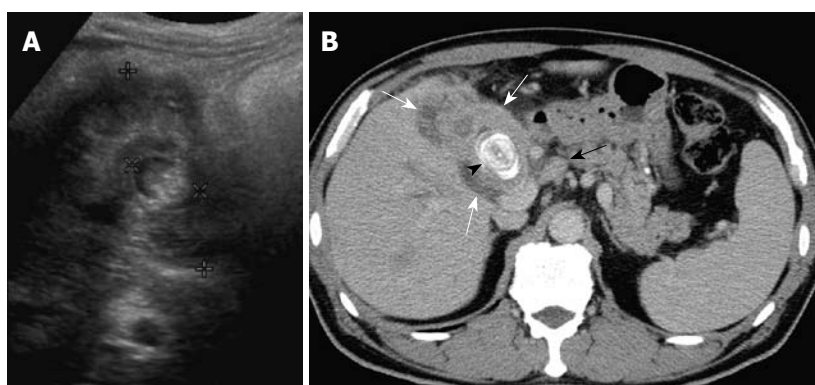


Figure 3 A 77-year-old woman with right upper abdominal pain and jaundice for 1 mo. A: US showed a large gallstone (x cursors) encased by the diffusely thickened gallbladder wall, with a heteroechoic appearance (+ cursors); B: Contrast-enhanced CT showed diffuse, uneven wall thickening of the gallbladder (white arrows), with a large laminated gallstone (arrowhead) and hepatoduodenal ligament lymph node metastasis (black arrow).

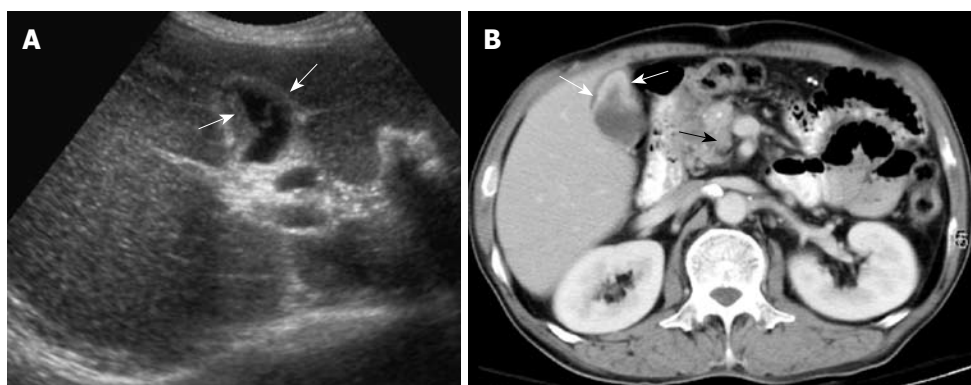


Figure 4 A 61-year-old man with dull right upper abdominal pain for 3 wk. A: US showed a relatively small gallbladder with focal wall thickening (arrows), which was misinterpreted initially as inadequate gallbladder distension; B: Contrast-enhanced CT was done because of persistent abdominal discomfort. It revealed relatively poor distension of the gallbladder with prominent enhancement of the focally thickened gallbladder wall (white arrows) and celiac lymph node metastasis (black arrow).

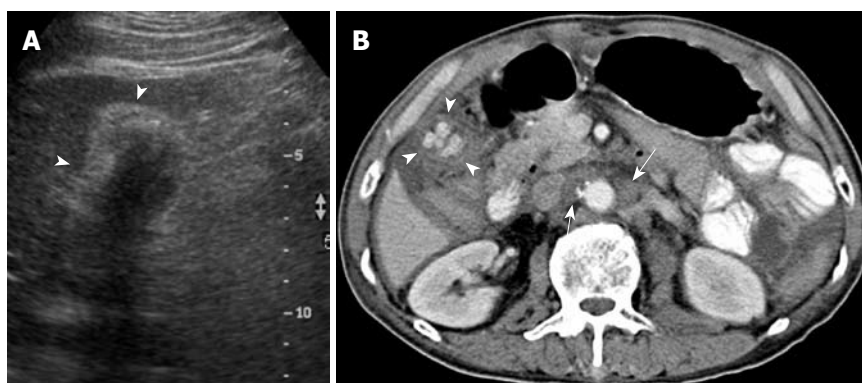


Figure 5 A 78-year-old man admitted to the emergency room with right upper abdominal pain, fever and jaundice for 1 d. A: US showed a contracted gallbladder with a thickened wall (arrowheads) and multiple gallstones that obliterated underlying details, which mimicked chronic cholecystitis; B: Contrast-enhanced CT showed a relatively small gallbladder with uneven wall thickening (arrowheads), multiple gallstones and pericholecystic inflammatory stranding and fluid suggestive of gallbladder carcinoma (GBCA), with coexistent acute cholecystitis. Note the intercavaortic and left para-aortic lymph node metastasis (white arrows).

sessile, and echogenic have been associated with a higher risk of malignancy, especially in patients > 60 years

old^[15,21-23]. The present study showed that the maximal diameter of intraluminal-GBCA varied from 1.1 to 3.8

cm (mean 2.3 cm), while the mean age of the patients was 65.5 years. Of note, five of our patients with intraluminal adenocarcinoma had no gallstones, while histopathological examination revealed tumor development from tubular or villotubular polypoid adenomas. Furthermore, all eight T1 tumors in the intraluminal-GBCA group had diameters between 1.1 and 1.4 cm. Therefore, our results supported prophylactic cholecystectomy of a single intraluminal polyp or mass > 1 cm in diameter^[15].

Although many physicians express a relatively nihilistic approach to the treatment of GBCA, several studies have encouraged an aggressive surgical approach that might lead to improved survival^[3-5]. Early detection and accurate preoperative diagnosis of GBCA is highly beneficial for surgical planning, especially in avoidance of laparoscopic surgery, which may induce recurrent tumors in the abdominal wall along the port track^[24,25]. Abdominal US and CT are the most common modalities for assessing suspected hepatobiliary malignancies^[6-11]. US allows the correct diagnosis in 70%-80% of advanced and 20%-30% of early gallbladder carcinoma cases^[6-9]. The present study disclosed that US was fairly sensitive for detecting intraluminal-GBCA with gallstones (sensitivity 63.6%) and highly sensitive for intraluminal-GBCA without gallstones (sensitivity 91.3%). Intraluminal-GBCA may appear as a hypoechoic polypoid lesion (> 1 cm) that projects into the lumen, a fungating mass, or a large mass that replaces the gallbladder, on US^[6-9]. In contrast, benign cholesterol or inflammatory polyps are usually multiple, hyperechoic and < 1 cm in diameter^[6,21-23]. Intraluminal-GBCA with a fungate appearance or large mass can be distinguished by a heterogeneous echotexture and irregular border of the lesions, as well as intralesional or perilesional echogenic foci and acoustic shadowing caused by coexisting gallstones and tumoral calcification^[7-9]. On the contrary, US has poor sensitivity for detecting infiltrating-GBCA, with and without gallstones (sensitivity of 12.5% and 25.0%, respectively). As demonstrated in the present study, possibly as a result of chronic gallbladder wall irritation by gallstones, and the infiltrating nature of the tumor, which might limit gallbladder expansion, the mean gallbladder size was significantly smaller in patients with infiltrating-GBCA than in those with intraluminal-GBCA. Subtle gallbladder wall thickening that leads to misinterpretation as chronic inflammatory changes, coexistence of acute cholecystitis and obliteration by concurrent gallstones or bile sludge are also factors that might hamper the US detection of infiltrating-GBCA^[6].

Similar to US, GBCA may appear as an intraluminal or infiltrating tumor upon CT^[7-9]. Our study showed that CT was 80% and 100% sensitive, respectively, for detecting intraluminal-GBCA with and without gallstones. In contrast to US, CT offered 71.4% and 75% sensitivity, respectively, for detecting infiltrating-GBCA with and without gallstones. Focal or diffuse gallbladder wall thickening (maximal thickness from 0.7 to 1.2 cm, mean 0.9 cm) is the most common CT finding. This finding is nonspecific and may occur in a variety of conditions, including extracholecystic inflammatory processes such as hepatitis, pancreatitis, pyelonephritis, adenomyomatosis, and chronic

or xanthogranulomatous cholecystitis^[24-27]. However, CT allows assessment of the condition of the liver, pancreas and kidney, and thus exclusion of secondary involvement of the gallbladder in extracholecystic inflammation is not difficult^[24,25]. CT also is helpful for revealing adenomyomatosis with proliferation of the subserosal fat and intramural diverticula with small calculi, chronic cholecystitis with a double-layered small gallbladder and a weakly enhancing thin inner layer, and xanthogranulomatous cholecystitis with intramural hypoattenuated nodules in a thickened gallbladder wall^[24-27].

Our study was limited by its retrospective nature. First, all patients had surgically proven GBCA, and thus, it was not possible to establish a false-positive rate for US and CT. A prospective study is needed to validate the accuracy, specificity, and true- and false-predictive values of US and CT for intraluminal and infiltrating-GBCA, but due to the infrequency of GBCA, such a large-scale prospective study may be difficult. Second, only a small proportion of our patients underwent percutaneous cholangiography, radionuclide and magnetic resonance imaging studies and hence, the role of these studies in these two morphologically different types of GBCA could not be determined.

In summary, in contrast to intraluminal-GBCA, infiltrating-GBCA is overlooked easily on US. In elderly women with suspected small gallbladder and gallstones upon US, especially those with jaundice, fever, and high serum alkaline phosphatase and total bilirubin levels, CT is helpful in surveying underlying infiltrating-GBCA.

COMMENTS

Background

Gallbladder carcinoma (GBCA) is a relatively uncommon cancer with early local invasion of the liver and biliary tree, but early diagnosis remains difficult because most patients present with nonspecific abdominal symptoms. Ultrasound (US) and computed tomography (CT) have been reported as helpful tools for imaging and staging of GBCA. To the best of our knowledge, the influences of different tumor morphology on clinical presentation, as well as on US and CT sensitivity of GBCA detection have not been well studied.

Research frontiers

US is the main initial diagnostic tool for suspected biliary lesions and may be helpful for detecting GBCA. GBCA may present as an intraluminal mass and an infiltrating tumor, and the infiltrative morphology of some tumors may render US assessment difficult. In addition, the presence of gallstones, inflammation and debris may preclude tumor detection by US. CT has been reported as a comprehensive tool for imaging and staging of GBCA, but its usefulness in patients with different tumor morphology has not been explored.

Innovations and breakthroughs

The results demonstrated that, compared with patients with intraluminal-GBCA, patients with infiltrating-GBCA were significantly older, and had a significantly higher frequency of jaundice and fever, higher alkaline phosphatase and total bilirubin levels, higher frequency of gallstones, smaller gallbladder size and shorter survival. The sensitivity for diagnosing intraluminal-GBCA with and without gallstones was 63.6% and 91.3%, respectively, by US, and 80% and 100%, respectively, by CT. The sensitivity for diagnosing infiltrating-GBCA with and without gallstones was 12.5% and 25%, respectively, by US, and 71.4% and 75%, respectively, by CT.

Applications

Intraluminal and infiltrating-GBCA exhibit different clinical presentations and features by US and CT. US and CT are fairly to highly sensitive for detecting intraluminal-GBCA with and without gallstones. Conversely, infiltrating-GBCA, especially when associated with gallstones, is overlooked easily by US. Further CT examination can

be useful in revealing underlying infiltrating-GBCA in elderly women with relatively small gallbladders and gallstones noted by US, particularly those with jaundice, fever, and high serum alkaline phosphatase and total bilirubin levels.

Terminology

Intraluminal-GBCA denotes tumor growths that protrude into the gallbladder lumen, which appear grossly as polypoid, fungating or large intraluminal masses. Infiltrating-GBCA denotes infiltrating carcinomas that appear grossly as focal or diffuse areas of wall thickening, or induration of the gallbladder wall.

Peer review

This is an interesting report of gallbladder cancer with differences in clinical presentation. Although it is of clinical significance, the presentation of the paper needs some modification.

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Inhibitory effect of emodin and Astragalus polysaccharide on the replication of HBV

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CONCLUSION: Emodin and APS have a weak but persistent inhibitory effect on HBV replication *in vivo*, which may function as a supplementary modality in the treatment of hepatitis B infection.

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Key words: Astragalus polysaccharides; Emodin; Hepatitis; Hepatitis B virus; Lamivudine

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Abstract

AIM: To evaluate the anti-viral effect of emodin plus Astragalus polysaccharide (APS) in hepatitis B virus (HBV) transgenic mice.

METHODS: Sixty HBV transgenic mice (HBV TGM) whose weight varied between 18 and 24 g were randomly divided into 3 groups, with 20 mice in each group. Group A was the normal control, where the mice were treated with physiological saline; group B was the positive control where the mice were treated with lamivudine solution (100 mL/kg per day). Group C was the experimental group where the mice were treated with physiological saline containing emodin and APS (57.59 mg/kg per day and 287.95 mg/kg per day, respectively). The mice were treated daily for 3 wk. After 1 wk recovery time, the mice were sacrificed and serum as well as liver tissues were collected for ELISA and histological examination.

RESULTS: After 21 d treatment, HBV DNA levels in group B and group C significantly declined when compared with group A ($P < 0.05$). However, a significant increase in HBV DNA content was observed in group B, whereas this phenomenon was not observed in group C. A reduction in the contents of HBsAg, HBeAg and HBcAg in the mice from group B and C was observed when compared with group A.

INTRODUCTION

Hepatitis B is a global health problem, and affects about 400 million people worldwide, particularly in Asian and Western Pacific countries^[1]. It is the leading cause of cirrhosis and liver cancer in China^[2]. The current treatment strategy for hepatitis B is to eradicate replication and infection of hepatitis B virus (HBV) *in vivo* using anti-virus drugs such as interferon and nucleoside analogs such as lamivudine^[3]. However, because of their side effects, low antiviral potency, and long treatment period, the actual effects of these treatments are neither ideal nor adequate^[4].

Chinese herbal medicine has been used for chronic liver diseases for thousands of years in China, and their efficacy has been confirmed by modern biological technology in recent years^[5,6]. Emodin (1,3,8-tri-hydroxy-6-methylanthraquinone) is an active component of herbal medicine derived from the genera *Rheum*, *Polygonum*, *Rhamnus* and *Senna*^[7]. Our previous study showed that emodin could inhibit the replication of HBV in human hepatoma cells *in vitro*^[8], suggesting its potential antiviral effect in hepatitis B treatment. Astragalus polysaccharide (APS) is a bioactive chemical in *Astragalus membranaceus* (AM) which has been used in medicine for centuries in China and is believed to exhibit immune-stimulatory, anti-viral, anti-oxidation and anti-tumor

effects^[9-12]. A previous study performed *in vivo* has shown that Astragalus-Polygonum Anti-Fibrosis Decoction, in which APS is one of the main components, significantly inhibited liver fibrosis induced by hepatitis B and suppressed hepatic inflammation^[13], illustrating that APS may be helpful in eradicating HBV replication *in vivo*.

In this study, we investigated the antiviral effects of emodin and APS in HBV transgenic mice and found that the combination of emodin and APS suppressed HBV replication in HBV transgenic mice. Although lamivudine had a stronger direct inhibitory effect on HBV replication, emodin and APS showed no HBV recurrence 7 d after the last treatment, suggesting a long-lasting effect and may prove to be a potential therapeutic modality for hepatitis B infections.

MATERIALS AND METHODS

Reagents

Emodin was purchased from Tianxingjian Bio. Co. (Xi'an, China), and was diluted to 5.80 mg/mL using dH₂O just before administration. APS was purchased from Hongsheng Biotech. Co. (Xi'an, China) and was diluted to 28.80 mg/mL in H₂O just before administration. Lamivudine was kindly provided by GlaxoSmithKline (GSK) China Co, and was diluted to 5 mg/mL in dH₂O.

Animals and drug administration

Sixty adult C57_TgN (HBVadr2.0) SMMU mice weighing between 18-24 g with an equal number of males and females, were provided by the Laboratory Animal Center and Department of Cell Biology of the Second Military Medical University. The mice were randomly divided into three groups with 20 mice in each group. Group A was the normal control, where the mice were administered physiological saline; group B was the positive control where the mice were administered lamivudine solution (100 mL/kg per day). Group C was the experimental group where the mice were administered physiological saline containing emodin and APS (57.59 and 287.95 mg/kg per day, respectively). The mice were treated daily for 3 wk followed by one week of recovery time without any treatment. The mice were then sacrificed. Blood was sampled from the abdominal aorta, centrifuged at 4°C, and plasma was stored at -20°C for assays; liver tissues were collected for histological examination.

Alteration in animal weight

The weight of each animal was measured just before the first drug administration and after sacrifice. The alteration in animal weight was calculated from these two values.

Histological examination

Hepatic tissues were fixed using 4% paraformaldehyde phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin wax. After deparaffinization, 5 μm sections were stained with hematoxylin and eosin (HE), and liver condition was classified according to the standard formulae published by the China Medical Association in 1995.

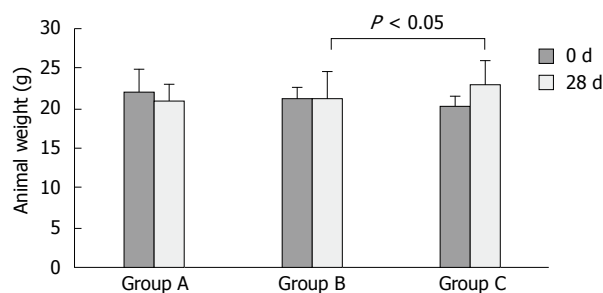


Figure 1 Animal weight alteration before and after the experiment. The weight of each mouse was determined before drug administration and before sacrifice after 28 d of experimentation, respectively. One-way ANOVA analysis indicated that there was no significant weight change between day 0 and day 28 in all three groups. However, increased weight was observed in group C compared with group B after 28 d of experimentation. Group A: Normal control; Group B: Lamivudine; Group C: Emodin and Astragalus polysaccharides.

HBsAg & HBcAg expression in tissue

The surface antigen of the hepatitis-B-virus (HBsAg) and hepatitis B core antigen (HBcAg) were detected using immunohistochemical staining as previously described^[13]. The stained slides were examined microscopically. Quantification of HBsAg and HBcAg positive cells was performed over several different areas of each section. The percentage of positive cells was evaluated by counting 100 cells (40 ×) in three consecutive tissue sections, and square scores were marked according to the following semi-quantitative criteria: 1: < 25% positive cells; 2: 26%-50% positive cells; 3: 51%-75% positive cells; 4: >75% positive cells. The intensity of the HBsAg staining within positive cells was evaluated, and the intensity scores were marked according to the following semi-quantitative criteria: 1: light yellow; 2: light brown; 3: chocolate brown. The final immunohistochemical reaction score (IRS) was calculated according to the formula IRS = positive staining scores × intensity scores.

HBsAg and HBeAg quantification

HBsAg and HBeAg (hepatitis B e antigen) levels in serum were determined using Enzyme-Linked Immunosorbent Assay (ELISA) (Huatai Biotechnologies Co, Shanghai, China) according to the manufacturer's instructions as previously described^[14].

HBV DNA concentration determination

Blood was collected *via* the abdominal aorta after the mice were sacrificed. After centrifugation at 8000 r/min for 5 min, the serum was separated and stored at -20°C. HBV DNA content in serum was determined using real-time PCR (PG Biotech Co, Shenzhen, China) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SE. Data was checked for normal distribution and equal variance. One-way ANOVA test analysis was carried out by SPSS 11.0 software. Differences were considered significant at *P* values of less than 0.05.

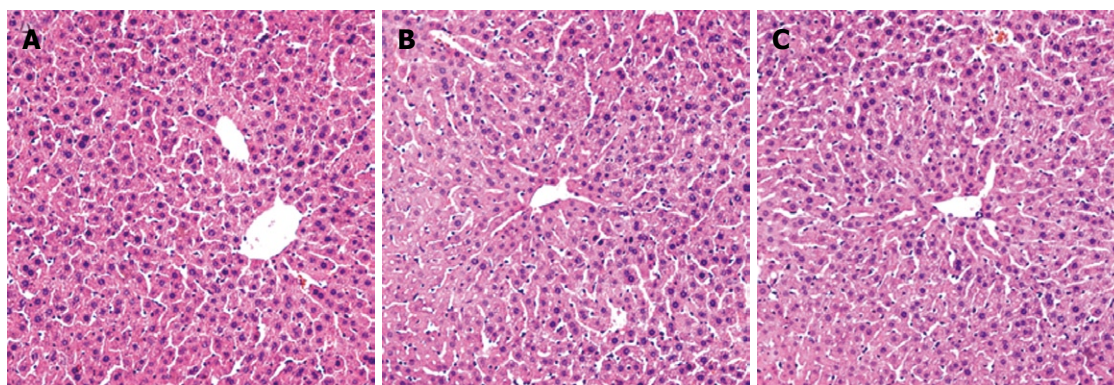


Figure 2 HE staining of liver tissue from different groups (Original magnification, $\times 200$). A: Normal control; B: Lamivudine; C: Emodin and Astragalus polysaccharides.

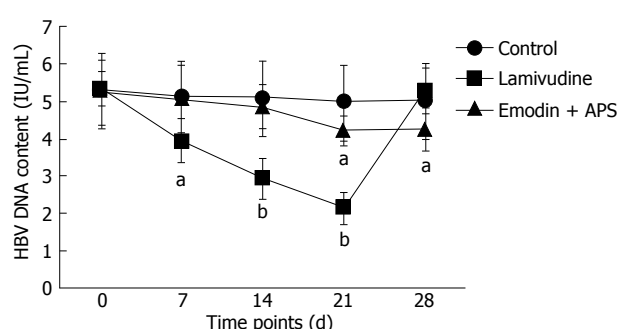


Figure 3 Hepatitis B virus (HBV) DNA levels in serum. Mice were sacrificed at day 28 of the experiment. Blood was collected, serum was separated, and HBV DNA content was investigated using real-time PCR. Lower HBV DNA contents were observed in the lamivudine group except on day 28 of the experiment. Emodin + APS decreased serum HBV DNA content at day 21 and 28. Data was presented as mean $\pm$ SE ($n = 19$). $^aP < 0.05$, $^bP < 0.01$ vs the control group at the same time point.

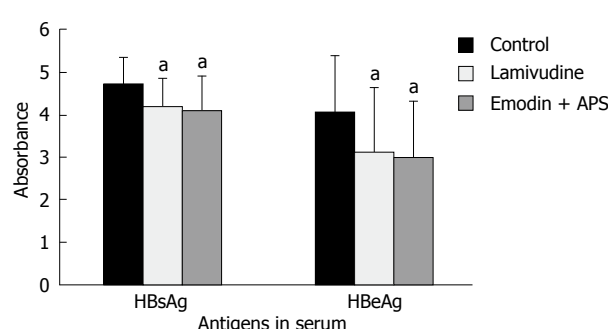


Figure 4 HBsAg and HBeAg levels in serum. Mice were sacrificed at day 28 of the experiment. Blood was collected and serum was separated. HBsAg and HBeAg levels in serum were determined using ELISA. Lower HBsAg and HBeAg levels were observed in the lamivudine and emodin + APS groups. Data was presented as mean $\pm$ SE ($n = 19$). $^aP < 0.05$ vs the control group.

RESULTS

Mice weight alteration

One mouse in group A died due to improper blood collection during the experiment at the third week. The other mice in all three groups were healthy during the experiments and their behaviour was normal. There was no significant weight alteration in the mice before or after the experiments. However, an increase in weight was observed in group C when compared with group B after 28 d of experimentation (Figure 1).

Histological examination

At the end of the experiment, there was no significant difference in the macro-appearance of the livers from mice in the three groups. The livers were pink, soft and their borders were even. Morphologically, the liver structure was intact, with few necrotic hepatocytes, limited inflammatory cell infiltration and fibrous tissue formation (Figure 2).

HBV DNA content in serum was reduced by lamivudine, emodin and APS

The use of real-time PCR showed that lamivudine significantly decreased serum HBV DNA content after one week of administration, and this inhibitory effect lasted up to 21 d. HBV DNA content increased to

the original level when lamivudine administration was stopped for one week. However, emodin and APS did not decrease serum HBV DNA content after 7 or 14 d of administration (Figure 3), but reduced HBV DNA content after 21 d of administration, and this inhibitory effect lasted up to day 28, one week after administration ceased (Figure 3). There was no alteration in serum HBV DNA content in mice from the control group.

Lamivudine and emodin + APS reduced HBsAg and HBeAg levels in serum

HBsAg and HBeAg levels in serum were determined using ELISA at day 28 of the experiment and showed that lamivudine and emodin + APS significantly decreased HBsAg and HBeAg levels in serum in the treated groups, compared with the control group ($P < 0.05$). However, there was no significant difference in HBsAg and HBeAg levels between the lamivudine group and the emodin + APS group ($P > 0.05$) (Figure 4).

Lamivudine and emodin + APS inhibited HBsAg and HBeAg expression in hepatocytes

HBsAg and HBeAg expression in mouse liver tissue was also investigated using immunohistochemistry. HBsAg and HBeAg positive staining was brown or dark brown, and mainly localised within the cytoplasm. Both HBsAg and HBeAg were distributed throughout the liver tissue,

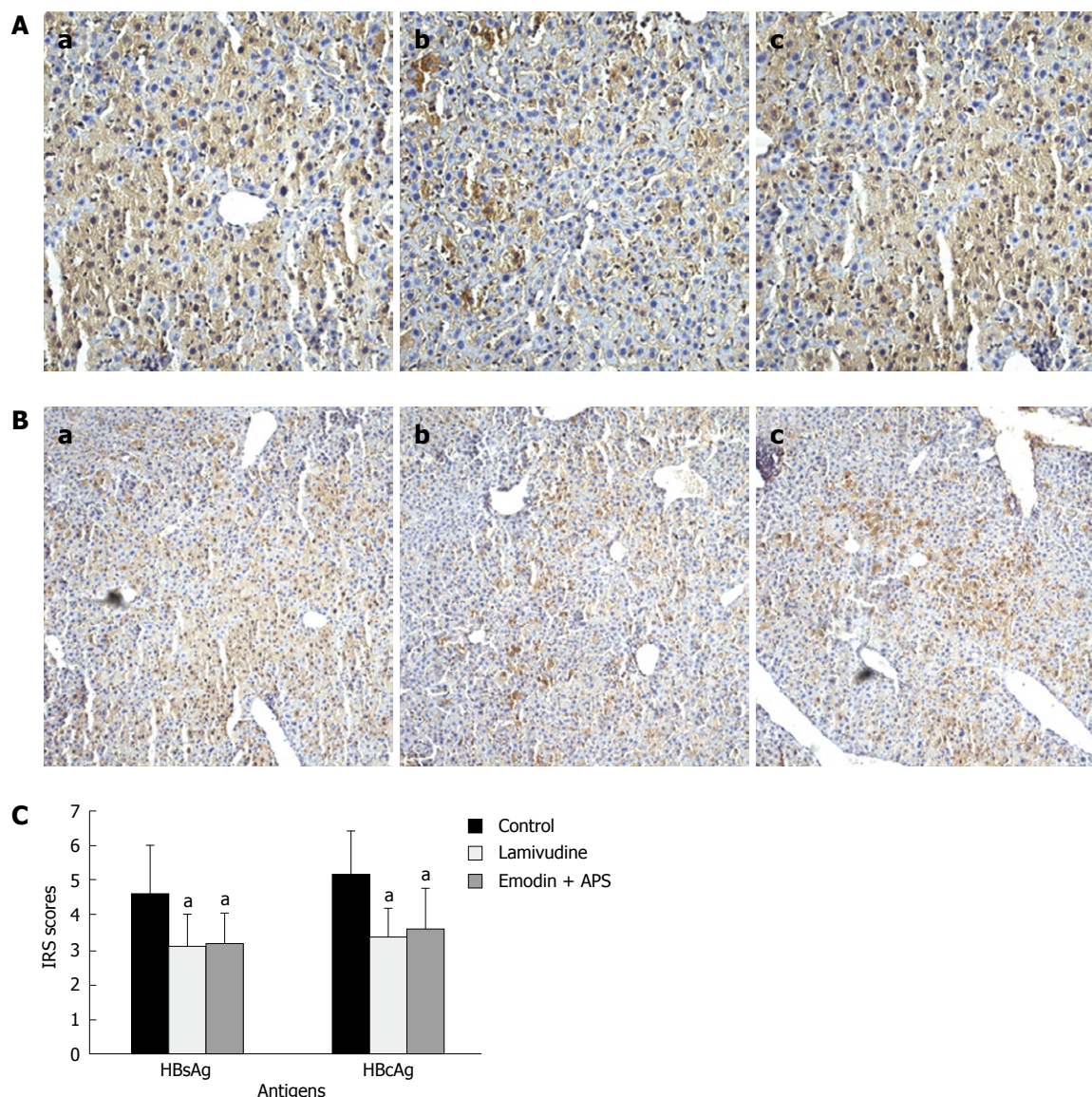


Figure 5 HBsAg and HBcAg expression in hepatocytes (original magnification, $\times 200$). Positive staining of HBsAg and HBcAg was brown or dark brown, and mainly localised in the cytoplasm. A: HBsAg; B: HBcAg; a: Control group; b: Lamivudine group; c: Emodin and Astragalus polysaccharides group; C: The immunohistochemical reaction score (IRS) was calculated according to the formula IRS = positive staining scores $\times$ intensity scores. Lower HBsAg and HBcAg expression were observed in the lamivudine and emodin + APS groups. Data was presented as mean $\pm$ SE. ^a $P < 0.05$ vs the control group.

especially in the portal area and around the central vein area (Figure 5A and B). The positive ratios of HBsAg staining in hepatocytes were 80% (control group), 75% (lamivudine group) and 80% (emodin + APS group), respectively, and the positive ratios of HBcAg staining were 55% (control group), 45% (lamivudine group) and 50% (emodin + APS group), respectively. There was no significant difference in the positive ratios of HBsAg and HBcAg staining in hepatocytes between these three groups ($P > 0.05$). However, lamivudine and emodin + APS decreased the positive staining of HBsAg and HBcAg in hepatocytes after analysis according to the IRS ($P < 0.05$) (Figure 5C).

DISCUSSION

The transgenic mouse model used in this study was established by integration of HBV genome into mouse genome using a microinjection method, and it has been

confirmed that HBV genes can be stably expressed, replicated and packaged in the mouse^[15]. The present study showed that administration of lamivudine for 3 wk significantly reduced serum HBV DNA content. However, after ceasing administration for 1 wk, HBV DNA returned to the original level. Similar results were obtained following administration of lamivudine to hepatitis B patients, suggesting that this transgenic mouse model can mimic HBV infection in man.

Using real-time PCR, we found that emodin + APS significantly reduced serum HBV DNA content, although this effect was weaker than that observed with lamivudine. Interestingly, the reduction in serum HBV DNA content in the emodin + APS group lasted longer, compared with the lamivudine group. This suggests that emodin + APS had a weaker but long-lasting antiviral effect on HBV.

HBV antigens including HBsAg, HBcAg and HBeAg are important markers for hepatitis B development in

patients^[16]. Our results indicate that emodin + APS significantly decreased the expression of these antigens in both serum and liver tissue and lamivudine had a stronger inhibitory effect. The mechanism of this inhibition is still largely unknown. Previous studies have shown that emodin can inhibit several different viruses such as herpes simplex virus (type I and II), parainfluenza virus and Coxsackie virus^[17-20]. More importantly, our previous study demonstrated that emodin can inhibit HBV replication *in vitro*^[8]. Therefore it is not surprising that emodin can inhibit HBV *in vivo*. APS has been used for chronic liver disease in China for thousands of years, and is known to increase CD3⁺ and CD4⁺ T-cells and the ratio of CD4⁺/CD8⁺ T-cells in mice, suggesting an immunoregulative effect^[10]. Therefore, the combination of emodin and APS not only resulted in inhibition of HBV replication, but also regulation of the immunologic system to eradicate HBV *in vivo*. This may explain the weaker and long-lasting effects of emodin + APS.

In conclusion, for the first time, we demonstrated that emodin and APS had a weak but long-lasting inhibitory effect on HBV replication *in vivo*, which may provide a new therapeutic option for hepatitis B infection.

COMMENTS

Background

The current treatment strategy for hepatitis B is to eradicate the replication and infection of hepatitis B virus (HBV) using anti-virus drugs such as interferon and nucleoside analogs such as lamivudine. However, the actual effects of these treatments are neither ideal nor adequate. The aim of this study was to evaluate the anti-viral effect of emodin plus Astragalus polysaccharide (APS) in HBV transgenic mice.

Research frontiers

In this study, the authors found that the combination of emodin and ASP suppressed HBV replication in HBV transgenic mice, which might be a potential alternative treatment method for HBV patients.

Innovations and breakthroughs

This is the first time that the combination of emodin and ASP was found to suppress HBV replication *in vivo*, which is valuable for anti-HBV drug screening in the future.

Applications

This study provides valuable experimental evidence for future anti-HBV drug studies. It may provide an alternative strategy for therapeutic intervention in the treatment of patients with HBV.

Terminology

Emodin (1,3,8-tri-hydroxy-6-methylantraquinone) is an active component of herbal medicine derived from the genera *Rheum*, *Polygonum*, *Rhamnus* and *Senna*. APS is a bioactive chemical in *Astragalus membranaceus* (AM) which has been used in medicine for centuries in China.

Peer review

This is a well performed experimental study showing for the first time a moderate effect on *in-vivo* HBV replication of two molecules derived from Chinese Traditional Medicine drugs.

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ORIGINAL ARTICLE

Nicotinamide overload may play a role in the development of type 2 diabetes

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diabetic and 14 non-diabetic subjects were compared using HPLC. Cumulative effects of nicotinamide and *N*¹-methylnicotinamide on glucose metabolism, plasma H₂O₂ levels and tissue nicotinamide adenine dinucleotide (NAD) contents of adult Sprague-Dawley rats were observed. The role of human sweat glands and rat skin in nicotinamide metabolism was investigated using sauna and burn injury, respectively.

RESULTS: Diabetic subjects had significantly higher plasma *N*¹-methylnicotinamide levels 5 h after a 100-mg nicotinamide load than the non-diabetic subjects ($0.89 \pm 0.13 \mu\text{mol/L}$ vs $0.6 \pm 0.13 \mu\text{mol/L}$, $P < 0.001$). Cumulative doses of nicotinamide (2 g/kg) significantly increased rat plasma *N*¹-methylnicotinamide concentrations associated with severe insulin resistance, which was mimicked by *N*¹-methylnicotinamide. Moreover, cumulative exposure to *N*¹-methylnicotinamide (2 g/kg) markedly reduced rat muscle and liver NAD contents and erythrocyte NAD/NADH ratio, and increased plasma H₂O₂ levels. Decrease in NAD/NADH ratio and increase in H₂O₂ generation were also observed in human erythrocytes after exposure to *N*¹-methylnicotinamide *in vitro*. Sweating eliminated excessive nicotinamide (5.3-fold increase in sweat nicotinamide concentration 1 h after a 100-mg nicotinamide load). Skin damage or aldehyde oxidase inhibition with tamoxifen or olanzapine, both being notorious for impairing glucose tolerance, delayed *N*¹-methylnicotinamide clearance.

CONCLUSION: These findings suggest that nicotinamide overload, which induced an increase in plasma *N*¹-methylnicotinamide, associated with oxidative stress and insulin resistance, plays a role in type 2 diabetes.

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Key words: Type 2 diabetes; Nicotinamide; *N*¹-methylnicotinamide; Insulin resistance; Oxidative stress; Liver; Sweat glands

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Abstract

AIM: To investigate whether nicotinamide overload plays a role in type 2 diabetes.

METHODS: Nicotinamide metabolic patterns of 14

Zhou SS, Li D, Sun WP, Guo M, Lun YZ, Zhou YM, Xiao FC, Jing LX, Sun SX, Zhang LB, Luo N, Bian FN, Zou W, Dong LB, Zhao ZG, Li SF, Gong XJ, Yu ZG, Sun CB, Zheng CL, Jiang DJ, Li ZN. Nicotinamide overload may play a role in the development of type 2 diabetes. *World J Gastroenterol* 2009; 15(45): 5674-5684 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5674.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5674>

INTRODUCTION

Type 2 diabetes, characterized by insulin resistance and oxidative stress^[1,2], has reached epidemic proportions not only among adults, but also among adolescents in the past few decades, which has led to the hypothesis that type 2 diabetes is a result of gene-environment (diet) interactions, because the human genome has not changed markedly in such a short time^[3-5]. However, what the environmental/dietary risk factors are and how they function remain unclear.

Nicotinamide, the amide of nicotinic acid, is the precursor for the coenzymes β -nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which function in many enzyme-catalyzed oxidation and reduction reactions. Therefore, nicotinamide homeostasis is vitally important for the body^[6]. In humans, excess nicotinamide is methylated, oxidized or hydroxylated to N^1 -methylnicotinamide, nicotinamide- N -oxide or 6-hydroxynicotinamide, respectively, and then N^1 -methylnicotinamide is further oxidized to the pyridones N^1 -methyl-2-pyridone-5-carboxamide (2Py) and N^1 -methyl-4-pyridone-3-carboxamide by aldehyde oxidase (AOX; EC 1.2.3.1). The major nicotinamide metabolites in human urine are 2Py and N^1 -methylnicotinamide^[7]. Although the nicotinamide metabolism pathway is well understood, however, the influence of the catabolic efficiency of excess nicotinamide on the body is not fully understood.

Since pellagra was found to be related to vitamin B₃ (niacin) deficiency in 1930s, more attention has been paid to the prevention of niacin deficiency. As a result, nicotinamide is used extensively as a food additive without having undergone full formal safety evaluation^[8], and the chronic effects of nicotinamide overuse are far from understood^[9]. Nicotinic acid and nicotinamide frequently are reported to impair glucose tolerance and induce insulin resistance^[10-12]. Recent evidence suggests that N^1 -methylnicotinamide is involved in Parkinson's disease^[9]. More importantly, the abrupt increase in prevalence of type 2 diabetes in the United States in the latter half of the 20th century^[13] occurred roughly in parallel with the increase in per capita niacin content^[14]. A similar situation occurred in China, in which food enrichment with niacin began in 1980s, followed by a sharp increase in the incidence of diabetes from 1% in 1980 to 5.5% in 2001^[15]. However, whether type 2 diabetes pathogenesis involves nicotinamide overload is undetermined.

The present study aimed to compare nicotinamide metabolic patterns between diabetic and non-diabetic

subjects, and to investigate the relationship between nicotinamide overload and insulin resistance. We found that diabetic subjects exhibited slow N^1 -methylnicotinamide clearance, and that N^1 -methylnicotinamide could trigger oxidative stress and insulin resistance, which suggested that the association of nicotinamide overload with relatively slow N^1 -methylnicotinamide detoxification and excretion might be at least partially responsible for the development of type 2 diabetes.

MATERIALS AND METHODS

Chemicals

Nicotinamide was purchased from Sigma (St. Louis, MO, USA). Nicotinamide tablets (50 mg/tablet) were purchased from Lisheng Pharma (Tianjin, China). N^1 -methylnicotinamide was purchased from Takeda Chemical Industries (Osaka, Japan). Tamoxifen was from Kunshan Sanyou Pharmaceutical Adjuvant Factory (Kunshan, China). Olanzapine was obtained from Beijing Nordhuns Chemical Technology (Beijing, China). N^1 -ethylnicotinamide and 2-Py were synthesized according to the methods described respectively by Hirayama *et al*^[16] and Holman *et al*^[17].

Nicotinamide load test in humans

This study was approved by the relevant ethics committee, and all the Chinese participants gave informed consent. Fourteen type 2 diabetic patients from eight families with a positive history (eight men and six women, mean age, 55.3 ± 10.2 ; range, 36-75 years) and 14 age- and sex-matched healthy volunteers without a family history (mean age, 53.8 ± 10.8 ; range, 39-75 years) participated in this study. Type 2 diabetes was defined as a fasting glucose level ≥ 7.0 mmol/L or current receipt of hypoglycemic medication. All subjects refrained from drugs, alcohol and caffeinated products for at least 12 h before the study. After an overnight fast, urine was collected and quantitated 1 h before, and 2, 4 and 5 h after loading with 100 mg nicotinamide. Venous blood was collected into sodium citrate tubes before and 5 h after nicotinamide loading, and separated by centrifugation (1500 g, 10 min). Aliquots of each plasma and urine sample were placed directly in liquid nitrogen and then transferred to -80°C and -20°C, respectively.

Cumulative nicotinamide and N^1 -methylnicotinamide experiments

All animal experiments were conducted in accordance with institutional guidelines. Male Sprague-Dawley rats (180-220 g) were fed a standard rat chow. In nicotinamide experiment, rats were divided randomly into three groups ($n = 6$ each) and fasted for 14 h before the experiment. In the two nicotinamide-treated groups, nicotinamide (100 or 400 mg/kg) was administered (intraperitoneally, ip) and repeated every 2 h for five doses. Glucose tolerance test was performed by injection of glucose (2 g/kg, ip) 2 h after the final nicotinamide injection. Blood glucose was measured 1 h after glucose injection. Blood was collected

by eye bleed into EDTA tubes under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose administration. Plasma was separated by centrifugation (1500 g, 10 min). After plasma collection, the buffy coat and top fifth reticulocyte-rich layer of erythrocytes were discarded. Aliquots of plasma and erythrocytes, and harvested samples of liver and gastrocnemius muscle were plunged directly into liquid nitrogen and subsequently stored at -80°C until assay. The same protocol was used in the N^1 -methylnicotinamide experiment, except that the two treated groups repeatedly received 100 or 400 mg/kg N^1 -methylnicotinamide per injection and a total dosage of 0.5 or 2 g, respectively (each group, $n = 6$).

AOX inhibition

Rats in inhibitor-treated groups received subcutaneous tamoxifen (50 mg/kg) or olanzapine (20 mg/kg) twice daily for 4 d, and rats in each control group received vehicle only (each group, $n = 6$). After an overnight fast, all the rats received nicotinamide (100 mg/kg, ip). Plasma samples were collected 5 h after nicotinamide administration as described above.

Chronic AOX inhibition

Rats were divided initially into two groups: tamoxifen-treated (20 mg/kg per day, subcutaneously, $n = 24$) and vehicle-treated (control, $n = 8$). Eight control and eight tamoxifen-treated rats were sacrificed at the end of 7 wk. Liver samples were harvested and stored at -80°C for western blotting. The remainder of tamoxifen-treated rats was then divided into two groups treated with tamoxifen (20 mg/kg per day) with or without N^1 -methylnicotinamide (100 mg/kg per day, subcutaneously), respectively. Two weeks after the treatment, glucose tolerance test was performed by injection of glucose (2 g/kg, ip) after an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose injection. Samples of plasma, liver and gastrocnemius muscle were harvested under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose injection.

Thermal injury

Rats in the burn group ($n = 11$) were given a 40% total body surface area, full-thickness scald burn under ether anesthesia by immersion of the back in 95°C water for 15 s, as previously described^[18]. Sham rats ($n = 7$) were subjected to an identical procedure, except that they were immersed in 25°C water. All rats received glucose (2 g/kg, ip) 24 h after burning, with an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose dosing. Samples of plasma, liver and gastrocnemius muscle were harvested 2 h after glucose injection.

Sweat collection

Five healthy young male volunteers aged 20-24 years participated in this study. After an overnight fast, whole body sweat was collected before and 1, 2 and 3 h after a single oral dose of 100 mg nicotinamide, by having the volunteers stay in a plastic bag during sauna treatment (80°C, 15 min), with stringent precautions to minimize

evaporative loss. Aliquots of sweat were placed in liquid nitrogen, and transferred to -80°C until analysis.

Assays of glucose, insulin and glycogen

Blood glucose was measured using a glucometer (OneTouch Ultra; LifeScan Inc.). Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). Muscle and liver glycogen contents were determined with Glycogen Assay Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

H₂O₂ assay

Blood from healthy adult male volunteers was collected in EDTA-containing tubes and centrifuged (1500 g, 10 min). The plasma, buffy coat, and top fifth reticulocyte-rich layer of erythrocytes were discarded, and the remaining cells were washed three times in isotonic saline, and centrifuged (1500 g, 10 min). After the supernatant was discarded, the packed erythrocytes were transferred to Earle's Balanced Salt Solution presaturated with 95% O₂ and 5% CO₂ to make an erythrocyte suspension (20 μ L packed erythrocytes/mL). Two hundred microliters of erythrocyte suspension was added to each well of a 96-well plate in the absence or presence of N^1 -methylnicotinamide or 2Py at concentrations of 10 nmol/L to 100 μ mol/L, and incubated for 3 h at 37°C. H₂O₂ concentrations in the supernatant of cell cultures and in rat plasma were measured using an H₂O₂ Assay Kit (Beyotime Biotechnology, Jiangsu, China).

NAD/NADH assay

Human erythrocyte suspension (20 μ L packed erythrocytes/mL Earle's Balanced Salt Solution) was incubated in 1.5-mL Eppendorf tubes with a 1.5-mm hole in the cover, for 4 h at 37°C in the presence or absence of N^1 -methylnicotinamide (10 μ mol/L). The tubes were centrifuged (1500 g, 15 min, 4°C) and the supernatant was discarded. Four hundred microliters of BioVision NAD/NADH Extraction Buffer (Mountain View, CA, USA) was added to each tube for 5 min to lyse the cells. The lysates were ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). Assays were performed using the NAD⁺/NADH Quantification Kits according to the manufacturer's instructions (BioVision). For rat tissue NAD/NADH assay, 20 mg frozen liver, 20 mg frozen muscle or 20 μ L packed erythrocytes were homogenized in 400 μ L BioVision NAD/NADH Extraction Buffer. The homogenate was ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). The assay was conducted following the manufacturer's instructions.

Western blotting

Western blotting analysis of AOX was performed according to standard protocols. Briefly, 100 μ g rat liver proteins were separated on 5%-8% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in PBS that contained 0.1% Tween-20 and 5% non-fat dry milk for

30 min at room temperature, and incubated with antibody to AOX (1:250; BD Transduction Laboratories, Lexington, KY, USA) overnight at 4°C. Then, the membranes were washed by PBS-Tween followed by 1 h incubation at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected using the enhanced chemiluminescence (ECL) (Amersham Life Science).

Determination of *N*¹-methylnicotinamide, nicotinamide and 2Py

*N*¹-methylnicotinamide, nicotinamide and 2Py were analyzed using an HPLC system that consisted of an LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector with a 20-μL sample loop (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA), a Waters 470 fluorescence detector (Milford, MA, USA) for *N*¹-methylnicotinamide measurements, and a UV3000 detector (Thermo Separations Products, Fremont, CA, USA) for 2Py detection. *N*¹-methylnicotinamide concentration was analyzed by detecting its fluorescent 1,6-naphthylridine derivatives according to the method of Musfeld *et al.*^[19], using 366 nm excitation and 418 nm emission wavelengths, with the mobile phase (10 mmol/L sodium heptanesulfonate, 50 mmol/L triethylamine and 22% acetonitrile in water, pH 3.2 with 85% H₃PO₄) at a flow rate of 1 mL/min. Nicotinamide was quantitated by the same procedure after quantitative conversion of nicotinamide to *N*¹-methylnicotinamide using iodomethane, according to the method of Clark^[20]. For analyzing urinary 2Py, 50 μL 85% H₃PO₄ and 0.45 mL water were added to each urine sample (0.5 mL). After brief vortex mixing, the samples were centrifuged (12000 *g*, 10 min). The supernatant was filtered through a 0.45-μm filter for HPLC analysis. Standard solutions were prepared for calibration, which contained 0, 0.1, 1, 10 and 100 μg/mL of pure 2Py in normal urine. The mobile phase (5% methanol, 10 mmol/L KH₂PO₄, 10 mmol/L triethylamine, pH 3.0 adjusted with 85% H₃PO₄) was delivered at 1 mL/min. UV detection was performed at 260 nm.

Statistical analysis

The data are presented as means ± SD. Statistical differences in the data were evaluated by paired or unpaired Student's *t* test, or ANOVA as appropriate, and were considered significant at *P* < 0.05.

RESULTS

Slow *N*¹-methylnicotinamide clearance is a prominent feature of type 2 diabetes

The 5-h total urinary 2Py excretion after 100 mg nicotinamide load in the diabetic group was significantly less than that in the non-diabetic group (20.9 ± 4.5 mg *vs* 24.3 ± 4.1 mg, *P* < 0.05, Figure 1A and C), but urinary *N*¹-methylnicotinamide excretion increased in the diabetic group (Figure 1B and D). The plasma *N*¹-methylnicotinamide level 5 h after nicotinamide load was significantly higher in the diabetic than non-diabetic

group (Figure 2). It should be noted that there were no statistical differences in the basal urinary excretions of 2Py and *N*¹-methylnicotinamide and the basal levels of plasma *N*¹-methylnicotinamide between the two groups (Figures 1 and 2). These results suggest that slow plasma *N*¹-methylnicotinamide clearance, which can be revealed by nicotinamide load test, may be a potential biomarker of type 2 diabetes.

High plasma *N*¹-methylnicotinamide induces insulin resistance

We examined the effect of nicotinamide overload on rat glucose metabolism. Rats treated with cumulative doses of nicotinamide (2 g/kg) exhibited significantly higher levels of blood glucose and plasma insulin, but significantly lower muscle glycogen content than control rats after glucose load (Figure 3A). Another notable change after nicotinamide administration was that there was a marked increase in plasma *N*¹-methylnicotinamide (Figure 3A), so we examined the effects of *N*¹-methylnicotinamide. Cumulative doses of *N*¹-methylnicotinamide (2 g/kg) had comparable effects to nicotinamide (Figure 3B), which suggested that the effects of nicotinamide overload might have been mediated by *N*¹-methylnicotinamide.

*N*¹-methylnicotinamide triggers oxidative stress

Type 2 diabetes is associated with oxidative stress^[1,2]. We therefore examined whether nicotinamide overload and high *N*¹-methylnicotinamide levels were implicated in oxidative stress. The results showed that cumulative effects of nicotinamide (2 g/kg) or *N*¹-methylnicotinamide (2 g/kg) led to a significant increase in rat plasma levels of H₂O₂ (Figure 4A and B), a major reactive oxygen species (ROS) and common indicator of oxidative stress^[2]. Such an enhancing effect was also observed in human erythrocytes *in vitro* at physiological concentrations of *N*¹-methylnicotinamide (Figure 4C), whereas 2Py, the end product of nicotinamide, did not have an enhancing effect at the observed concentrations (10 nmol/L to 100 μmol/L) (data not shown). These results indicate that high plasma *N*¹-methylnicotinamide may induce systemic oxidative stress.

Increasing evidence has indicated that type 2 diabetes has abnormalities in the NAD⁺/NADH redox couple^[21]. We therefore investigated whether *N*¹-methylnicotinamide affected tissue NAD levels. Cumulative exposure to *N*¹-methylnicotinamide significantly reduced rat muscle and liver NAD (NAD⁺ + NADH) contents (Figure 4D and E). Notably, the erythrocytes of rats treated with cumulative doses of *N*¹-methylnicotinamide (2 g/kg) exhibited a significant increase in NADH and decrease in NAD/NADH ratio (Figure 4F). A similar effect was observed in human erythrocytes *in vitro* (Figure 4G). These results suggest that *N*¹-methylnicotinamide-induced oxidative stress may originate from imbalance in the NAD⁺/NADH redox couple.

AOX inhibition reduces *N*¹-methylnicotinamide clearance

AOX is responsible for conversion of *N*¹-methylni-

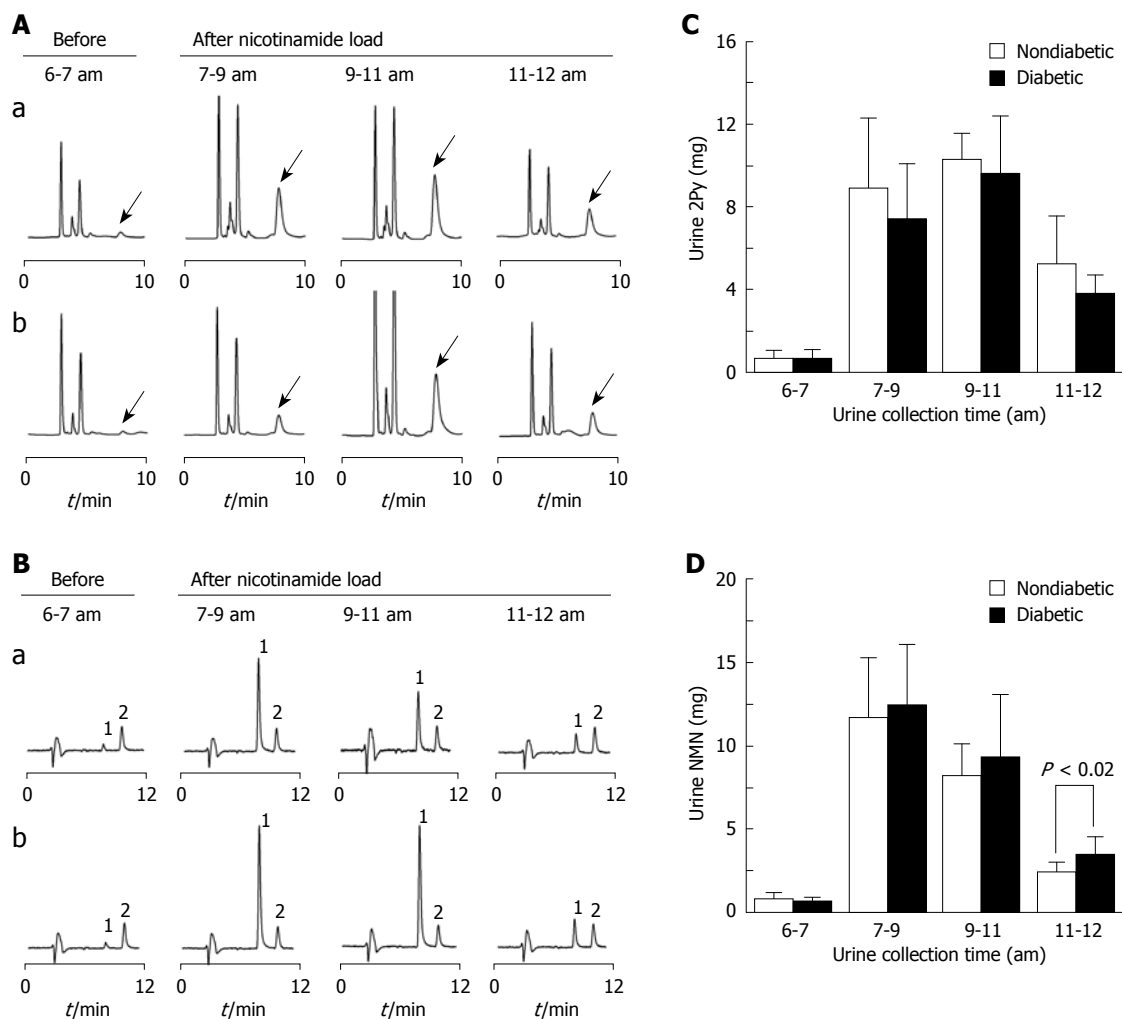


Figure 1 Urinary excretion patterns of 2Py and *N*¹-methylnicotinamide in diabetic and non-diabetic subjects. A and B: Representative HPLC chromatograms of urinary excretions of 2Py (indicated by arrow) and *N*¹-methylnicotinamide (NMN) of a non-diabetic (Aa and Ba) and a diabetic (Ab and Bb) subject, before and after 100 mg nicotinamide loading at 7:00 am. Urine samples taken at given time were normalized to equal volumes before HPLC analysis; C and D: Summaries of the results from the measurements shown in A and B, respectively. Bar graphs indicate mean $\pm$ SD.

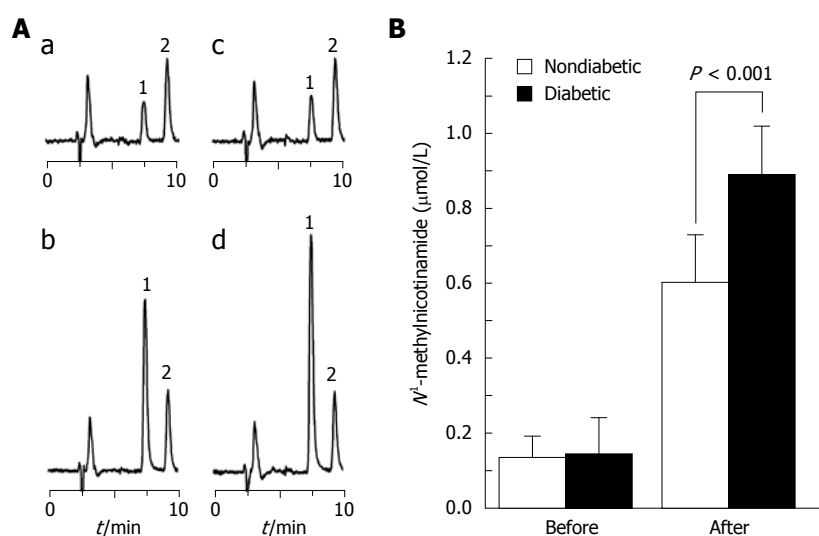


Figure 2 Plasma *N*¹-methylnicotinamide levels of diabetic and non-diabetic subjects after nicotinamide loading. A: Representative HPLC chromatograms of plasma *N*¹-methylnicotinamide levels from a non-diabetic (a, b) and a diabetic (c, d) subject before (a, c) and 5 h after (b, d) 100 mg nicotinamide loading. 1: *N*¹-methylnicotinamide; 2: Internal standard: *N*¹-methylnicotinamide; B: Summary of the results from the measurements shown in A. Bar graph indicates mean $\pm$ SD.

cotinamide-to-2Py^[22]. We examined changes in *N*¹-methylnicotinamide clearance after rat AOX inhibition by the putative inhibitors tamoxifen and olanzapine^[23], both of which are known to impair glucose tolerance^[24,25].

The rats treated with tamoxifen (100 mg/kg per day) or olanzapine (40 mg/kg per day) for 4 d exhibited significantly higher plasma *N*¹-methylnicotinamide levels than control rats 5 h after 100 mg/kg nicotinamide

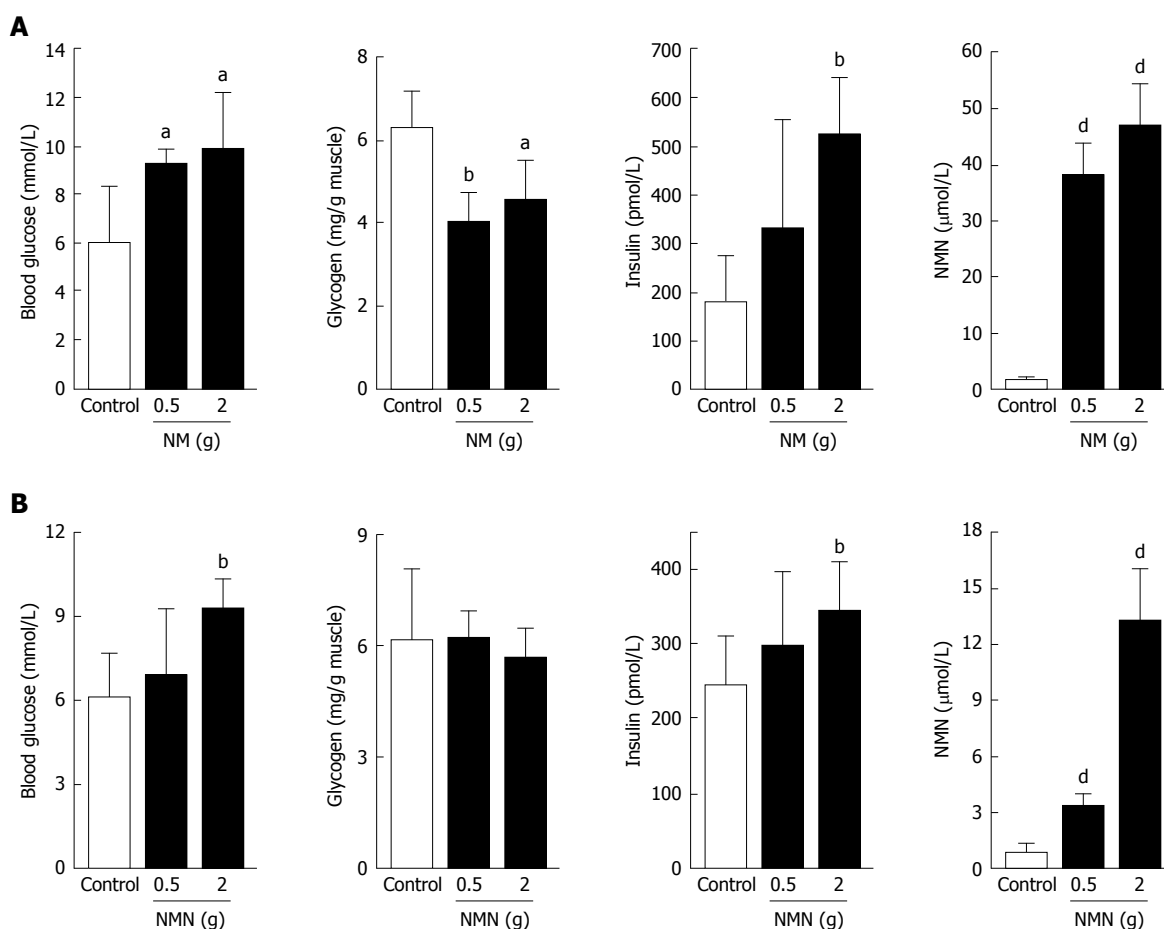


Figure 3 Effects of nicotinamide and *N*¹-methylnicotinamide on glucose metabolism of rats. A: Changes in blood glucose, muscle glycogen, plasma insulin and plasma *N*¹-methylnicotinamide in rats treated with or without cumulative nicotinamide (0.5 or 2 g/kg) after glucose loading; B: Comparable effects of cumulative *N*¹-methylnicotinamide (0.5 or 2 g/kg). NM: Nicotinamide; NMN: *N*¹-methylnicotinamide. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^d*P* < 0.001 vs control. Bar graphs show mean ± SD.

loading (Figure 5A and B). Moreover, chronic tamoxifen treatment for 7 wk significantly reduced rat liver AOX protein expression (*P* < 0.05, Figure 5C). To further explore the role of nicotinamide overload in insulin resistance, we used tamoxifen plus *N*¹-methylnicotinamide to mimic the conditions of relatively low AOX activity and excess nicotinamide intake. Rats treated with tamoxifen plus *N*¹-methylnicotinamide exhibited significantly higher blood glucose and much lower liver glycogen content than those treated with tamoxifen alone after glucose loading (Figure 5D).

Skin involvement in *N*¹-methylnicotinamide-mediated insulin resistance

If *N*¹-methylnicotinamide is indeed involved in insulin resistance, then any factors related to *N*¹-methylnicotinamide detoxification and excretion may affect insulin sensitivity. Of the factors, skin may be of particular significance because it takes part in *N*¹-methylnicotinamide detoxification and excretion, respectively, through skin AOX^[26,27] and sweat glands^[28]. We thus explored the role of skin by analyzing human sweat excretion of nicotinamide and *N*¹-methylnicotinamide after nicotinamide loading, or by assessing changes in plasma *N*¹-methylnicotinamide

clearance of rats with severe skin damage. Importantly, we found that nicotinamide concentrations in the sweat 1 h after 100 mg nicotinamide loading was 5.3-fold higher than that in fasting sweat, whereas *N*¹-methylnicotinamide concentrations were not significantly altered under such conditions (Figure 6A and B). This indicates that excess nicotinamide, without needing any conversion, is expelled through sweat. Moreover, this study found that rats with severe skin damage had a significant elevation in plasma *N*¹-methylnicotinamide associated with high blood glucose and plasma insulin levels, but lower muscle glycogen content after glucose loading (Figure 6C).

DISCUSSION

The main findings of this study were that: (1) nicotinamide overload elevates plasma levels of *N*¹-methylnicotinamide associated with oxidative stress and insulin resistance; (2) the skin plays an important role in expelling excess nicotinamide and detoxifying *N*¹-methylnicotinamide; and (3) diabetic subjects have slow plasma *N*¹-methylnicotinamide clearance. These findings may contribute to explain the mechanism of oxidative stress and insulin resistance in a variety of clinical conditions.

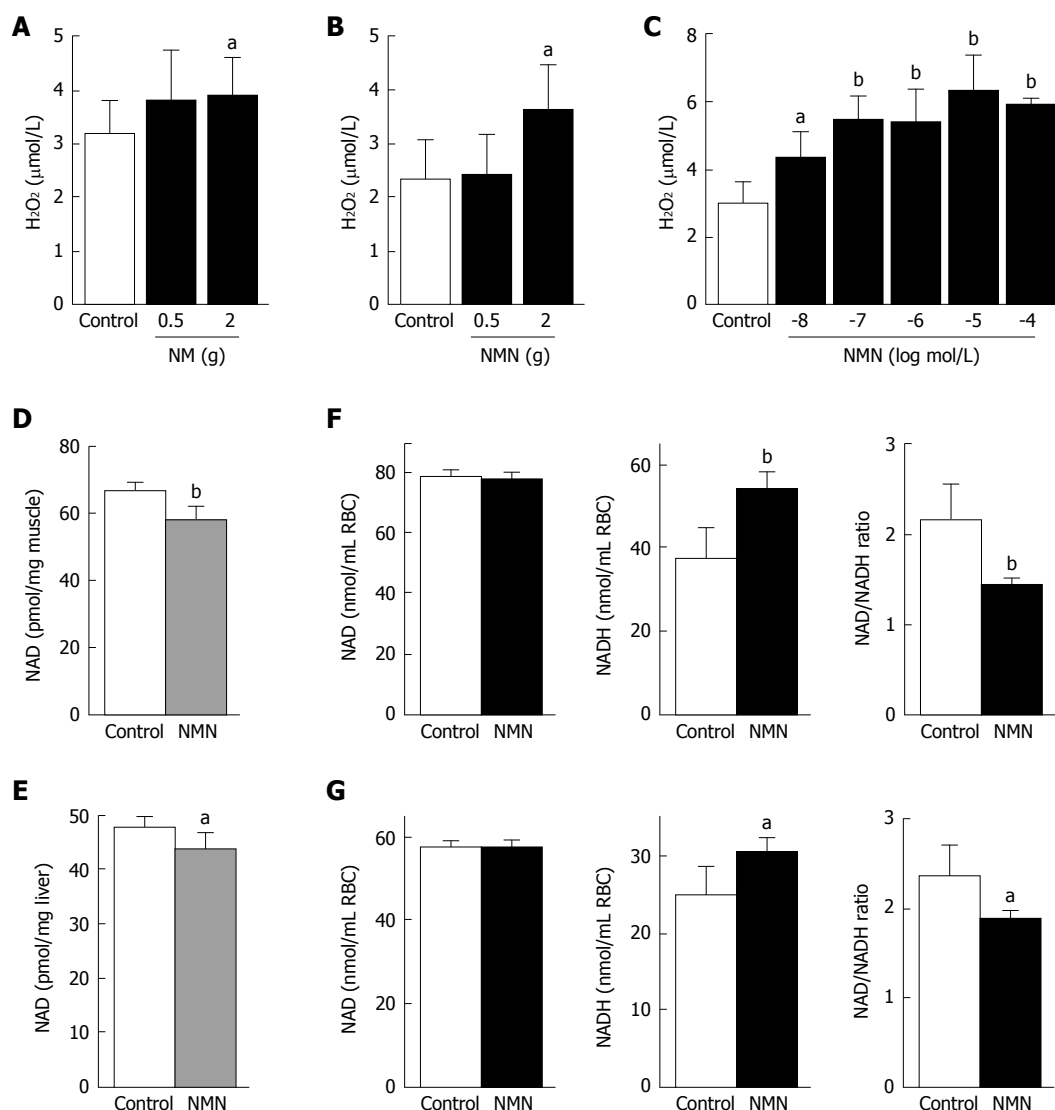


Figure 4 Effects of *N*¹-methylnicotinamide on H₂O₂ generation and NAD levels. A, B: Cumulative effects of nicotinamide (NM, 0.5 or 2 g/kg) and *N*¹-methylnicotinamide (NMN, 0.5 or 2 g/kg) on rat plasma H₂O₂ levels; C: H₂O₂ concentrations in the supernatant of cultured human erythrocytes with or without 3 h exposure to different concentrations of NMN. For each concentration, *n* = 4; D, E: NAD (NAD⁺ and NADH) contents in muscle (D) and liver (E) of rats treated with saline (control) or a cumulative dose of 2 g/kg NMN; F: NAD and NADH contents and NAD/NADH ratio in the erythrocytes (RBCs) of rats with or without cumulative exposure to NMN; G: NAD and NADH contents, and NAD/NADH ratio in human RBCs with (*n* = 4) or without (control, *n* = 4) 4 h exposure to 10 μmol/L NMN *in vitro*. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control. Bar graphs indicate mean ± SD.

*N*¹-methylnicotinamide is a potent trigger of oxidative stress and insulin resistance

Insulin resistance and oxidative stress are the major hallmarks of type 2 diabetes^[1,2], but the mechanisms underlying the development of systemic oxidative stress and insulin resistance are unclear. Both nicotinic acid and nicotinamide have been reported to induce insulin resistance, which may lead to elevation of plasma insulin due to β-cell compensation^[10-12]. Coincidentally, our data showed that nicotinamide overload induced acute insulin resistance in rats associated with high plasma levels of *N*¹-methylnicotinamide; the methylation product of nicotinamide being more toxic than nicotinamide (> 6-fold)^[29]. Importantly, diabetic subjects exhibited significantly higher plasma *N*¹-methylnicotinamide levels than non-diabetic subjects after nicotinamide loading, which suggests its involvement in the toxic effects of nicotinamide overload. Indeed, this study

demonstrated that *N*¹-methylnicotinamide mimicked the effect of nicotinamide overload, which suggested *N*¹-methylnicotinamide mediation of the toxic effect.

Increasing evidence suggests that insulin resistance is due to an unfavorable internal environment because muscles resistant to insulin, when cultured *in vitro*, regain sensitivity to insulin^[30-32]. Further evidence reveals that systemic oxidative stress may be responsible for triggering insulin resistance^[1,33]. Consistent with previous research, this study found that *N*¹-methylnicotinamide not only elevated plasma H₂O₂ levels *in vivo*, but also directly stimulated H₂O₂ generation of human erythrocytes *in vitro* at physiological concentrations, which indicates that *N*¹-methylnicotinamide is a potent trigger of diabetic oxidative stress.

Oxidative stress may induce NAD depletion, a marker of cell injury^[34,35]. Indeed, this study found that *N*¹-methylnicotinamide-induced high plasma H₂O₂ level was associated with a significant reduction in NAD content in

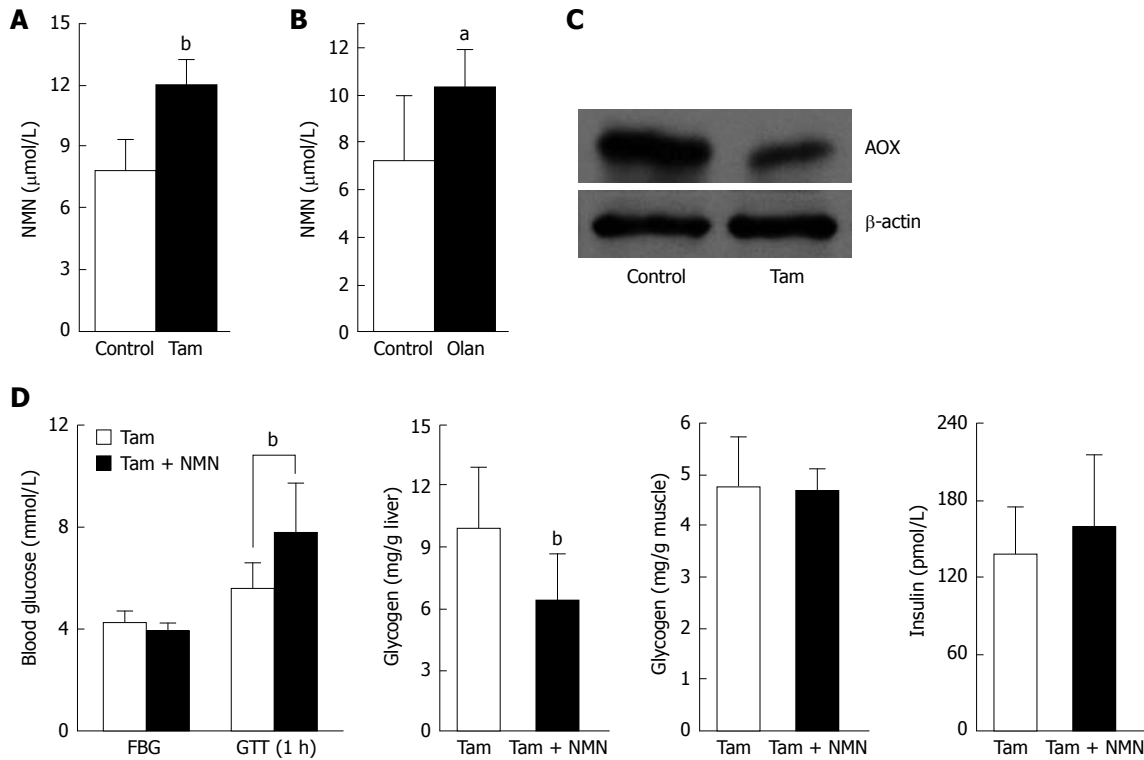


Figure 5 Effects of aldehyde oxidase (AOX) inhibitors on plasma *N*¹-methylnicotinamide levels and glucose metabolism in rats. A and B: Plasma *N*¹-methylnicotinamide (NMN) levels 5 h after nicotinamide load (100 mg/kg, ip) in rats treated with or without AOX inhibitors tamoxifen (Tam, A) or olanzapine (Olan, B) (each group, *n* = 6). ^aP < 0.05 vs control, ^bP < 0.01 vs control; C: Liver AOX expression in rats with or without 7 wk tamoxifen treatment. The blot is representative of four independent experiments; D: Responses to a glucose tolerance test in rats after 9 wk treatment with tamoxifen with or without NMN (100 mg/kg per day) treatment in the last 2 wk. FBG: Fasting blood glucose; GTT (1 h): Blood glucose measured 1 h after glucose tolerance test. ^bP < 0.01 vs control. Bar graph indicates mean ± SD.

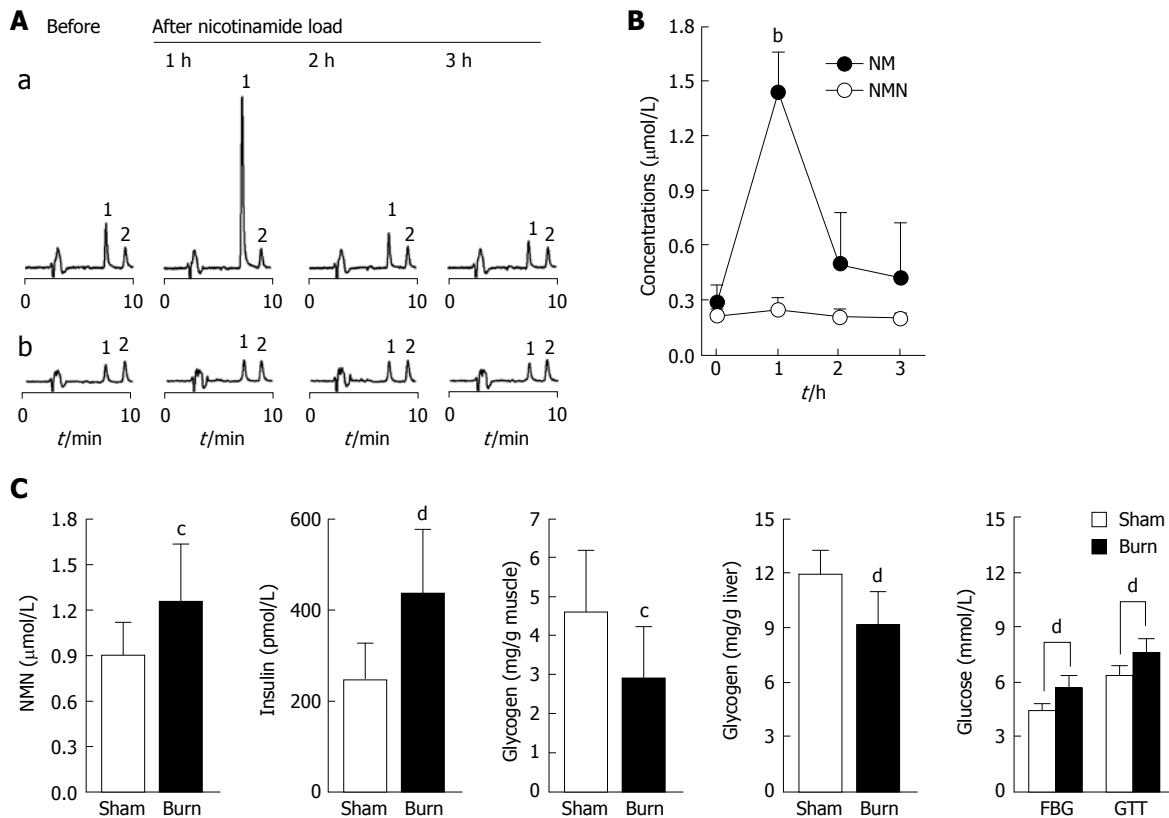


Figure 6 Role of skin in nicotinamide metabolism and insulin resistance. A: Representative HPLC chromatograms showing changes of sweat nicotinamide (NM) and *N*¹-methylnicotinamide (NMN) concentrations in a subject before and 1, 2 and 3 h after 100 mg nicotinamide loading. 1 and 2 in Aa are NM and internal standard *N*¹-ethylnicotinamide, respectively; 1 and 2 in Ab are NMN and internal standard *N*¹-ethylnicotinamide, respectively; B: Summary of the measurements shown in A. ^bP < 0.0001 vs control; C: Comparison of plasma NMN and insulin levels, muscle and liver glycogen contents, and blood glucose between sham-burn (*n* = 7) and burn (*n* = 11) rats after glucose load. FBG: Fasting blood glucose; GTT: Blood glucose 1 h after glucose injection. ^cP < 0.05, ^dP < 0.01 vs control. Bar graphs show mean ± SD.

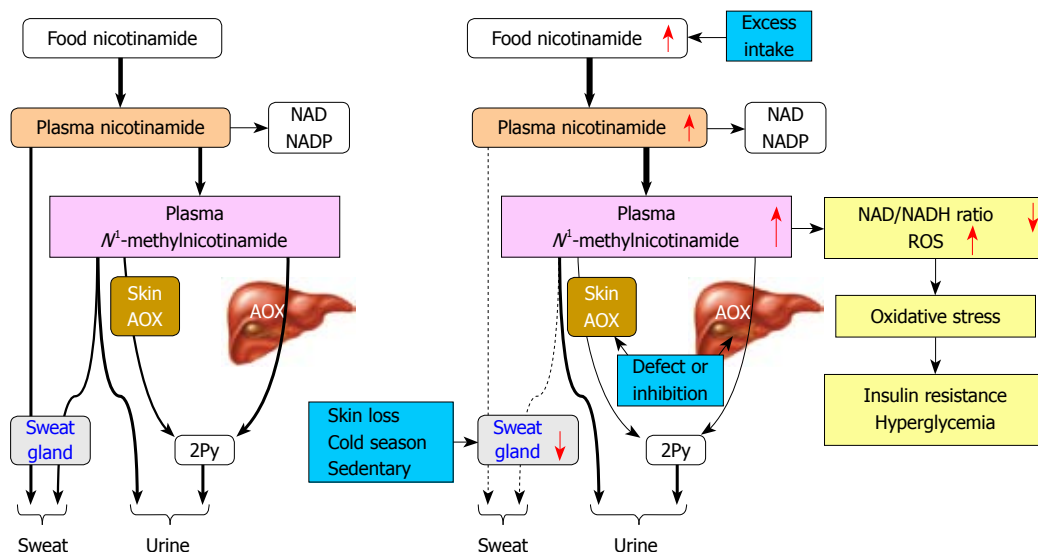


Figure 7 Proposed model of the role of nicotinamide overload in the development of type 2 diabetes. Normally, if nicotinamide intake is slightly more than the body needs, excess nicotinamide will be detoxified rapidly and eliminated mainly via the N^1 -methylnicotinamide to 2Py pathway, which involves liver and skin functions (left). Frequent excess nicotinamide intake, low N^1 -methylnicotinamide detoxification, or sweat gland inactivity induces a substantial rise in plasma N^1 -methylnicotinamide concentrations and residence time after each meal, and consequently induces oxidative stress and insulin resistance (right). The change trends are indicated by red arrows or line thickness.

the muscle and liver of rats. Then came the vital question: where did the excess systemic ROS originate? NADH-dependent ROS generation is an important source of intracellular ROS^[34]. Accumulating evidence has indicated that diabetes shows a decreased cytosolic $NAD^+/NADH$ ratio in a variety of tissues^[21]. Importantly, this study demonstrates that N^1 -methylnicotinamide decreases $NAD^+/NADH$ ratio in rat erythrocytes *in vivo* and human erythrocytes *in vitro*. Thus, it is likely that diabetic oxidative stress is initiated by high plasma N^1 -methylnicotinamide-induced imbalance in the $NAD^+/NADH$ redox couple. Many cellular processes are governed by the enzymes using $NAD^+/NADH$ as a cofactor^[6,21,34], therefore, it is not difficult to understand why a set of metabolic abnormalities happen in type 2 diabetes.

Role of AOX in insulin resistance

Mammalian AOX is a molybdo-flavo enzyme involved in the detoxification of various endogenous and exogenous N -heterocyclic compounds^[22,23]. N^1 -methylnicotinamide is one of the substrates of AOX by which N^1 -methylnicotinamide is oxidized to pyridones^[7], and thus, detoxified. AOX is expressed predominantly in the liver. Therefore, severe liver disease might be expected to reduce plasma N^1 -methylnicotinamide clearance and subsequent insulin resistance. In fact, it is well known that liver cirrhosis is associated with high incidence of diabetes^[36,37]. Pumpo *et al*^[38] have found that cirrhotic patients have high serum N^1 -methylnicotinamide levels, both in basal values and after nicotinamide loading. Moreover, non-alcoholic steatohepatitis, the most prevalent liver disease in the western world, typically is associated with the metabolic syndrome that is characterized by insulin resistance, diabetes and hypertension^[24]. Tamoxifen, a well-known inducer of non-alcoholic steatohepatitis^[24], is found to inhibit strongly AOX activity^[23] and its expression (Figure 5C). Collectively,

it seems that the decrease in N^1 -methylnicotinamide detoxification may be involved in hepatogenic insulin resistance.

AOX is also expressed in the skin^[26], which suggests skin involvement in N^1 -methylnicotinamide detoxification. Moreover, as found in this study, human sweat glands can excrete excess nicotinamide. Therefore, decreased skin function may be implicated in N^1 -methylnicotinamide-induced insulin resistance. In fact, severe burns may induce long-lasting insulin resistance, a well-documented but poorly understood phenomenon^[31,39]. The present study demonstrated that severe burns significantly delayed N^1 -methylnicotinamide clearance, which suggests that long-lasting post-burn insulin resistance may involve a decrease in nicotinamide detoxification and excretion.

Relationship between lifestyle risk factors and nicotinamide overload

Environmental and lifestyle risk factors may trigger type 2 diabetes^[3,4]. From our study, it emerges that the risk factors may involve nicotinamide overload. Firstly, excess nicotinamide may lead to high plasma N^1 -methylnicotinamide, with subsequent oxidative stress and insulin resistance. In response to insulin resistance, the pancreatic β cells have to increase insulin secretion (hyperinsulinemia) compensatorily, which may eventually lead to β -cell failure and blood sugar levels being out of control. The result that tamoxifen-induced AOX inhibition plus N^1 -methylnicotinamide impaired rat glucose tolerance (Figure 5D) implies that excess nicotinamide intake may be more harmful to those with a low N^1 -methylnicotinamide clearance.

Secondly, dietary risk factors may be related to nicotinamide contents in foods. For example, meat, which is rich in nicotinamide, increases the risk of type 2 diabetes^[40,41]. Moreover, food fortification with niacin may play a role in nicotinamide overload. If comparing the epidemic of type

2 diabetes in the United States^[13] with the history of food fortification^[14], one can clearly see that the trend for rapid increase in the incidence of diabetes has occurred in parallel with the trend in mandatory niacin-fortification-induced increase in the per capita niacin consumption in the latter half of the 20th century. These facts may explain why the western dietary pattern, characterized by a high intake of meat and niacin-fortified foods, confers such a high risk of type 2 diabetes.

Thirdly, sedentary lifestyle risk factor may involve sweat gland inactivity. Sweat gland activity fluctuates according to ambient temperature; the most significant feature of the gland. Therefore, sweat gland inactivity is expected to slow nicotinamide catabolism and thereby increase the danger of developing insulin resistance. In fact, the cold season is known to worsen glucose metabolism^[42,43], whereas exercising sufficiently to sweat may reduce diabetic incidence^[44]. Therefore, it is likely that sedentary lifestyle risk factors may be at least partially due to sweat gland inactivity by air-conditioned working/living environments.

It should be noted that large doses of nicotinic acid and nicotinamide may induce liver damage^[45,46], therefore, long-term investigation may be necessary to determine the relationship between chronic nicotinamide overload and non-alcoholic steatohepatitis. Historically, the epidemic of pellagra has been restricted mainly to those who have performed heavy industrial labor with poor nutrient supply^[9]. Hence, the present study gives rise to an important social and public health issue as to whether foods need to be fortified with niacin (nicotinamide or nicotinic acid), when the people in developed countries have already been living in an age of over-nutrition and sweat gland inactivity.

In summary, it appears that gene-environment (diet) interactions may be a reflection, to some extent, of the outcome of combination of nicotinamide overload and relatively low N^1 -methylnicotinamide detoxification and excretion. As summarized in Figure 7, the pathogenesis of type 2 diabetes may be at least partially due to long-term excess nicotinamide intake, and/or slowness in N^1 -methylnicotinamide detoxification, and/or decrease in excess nicotinamide and N^1 -methylnicotinamide excretion. This may lead to high plasma N^1 -methylnicotinamide levels, and subsequently oxidative stress and insulin resistance. Therefore, reducing nicotinamide intake and facilitating excretion of nicotinamide metabolites may be a useful preventive and therapeutic intervention in type 2 diabetes.

COMMENTS

Background

Type 2 diabetes generally is accepted to be a result of gene-environment interaction, although the underlying mechanism is not clear. Of the environmental factors, diet appears to play a major role. In fact, the sharp increases in the incidence of diabetes in the United States in the latter half of the 20th century and in China in the past two decades of the 20th century followed food fortification with niacin (i.e. nicotinamide and nicotinic acid) beginning in the early 1940s in the United States and in the early 1980s in China. Moreover, niacin is reported frequently to impair glucose metabolism and cause liver injury. Thus, there is the possibility that the high prevalence of type 2 diabetes in these countries in the past few decades may involve niacin toxicity.

Research frontiers

Type 2 diabetes is associated with increased systemic oxidative stress, a

factor responsible for the development of insulin resistance. How the systemic oxidative stress occurs is a major issue in type 2 diabetes.

Innovations and breakthroughs

The present study demonstrated that the pathogenesis of type 2 diabetes may involve abnormal nicotinamide metabolism. Factors that induce nicotinamide overload and/or decrease in the detoxification and excretion of nicotinamide metabolites may lead to systemic oxidative stress and insulin resistance. The factors may include the frequent use of foods rich in nicotinamide and/or fortified with niacin, congenital enzymatic defects, liver diseases, and sweat gland loss or inactivity. The present study suggests that gene-environment interactions may reflect the outcome of increased nicotinamide intake and a decrease in its detoxification and excretion.

Applications

Reducing nicotinamide intake and facilitating the excretion of excess nicotinamide may be a useful preventive and therapeutic intervention in type 2 diabetes.

Peer review

This is an interesting study with human and experimental data, which investigated a clinically relevant issue, and gave an insight into the pathogenic mechanisms involved. The experiments were performed well and are presented well in the paper.

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Capsule endoscopy in suspected small bowel Crohn's disease: Economic impact of disease diagnosis and treatment

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of suspected CD is comparable to SBFT and may be used immediately following ileo-colonoscopy.

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Abstract

AIM: To model clinical and economic benefits of capsule endoscopy (CE) compared to ileo-colonoscopy and small bowel follow-through (SBFT) for evaluation of suspected Crohn's disease (CD).

METHODS: Using decision analytic modeling, total and yearly costs of diagnostic work-up for suspected CD were calculated, including procedure-related adverse events, hospitalizations, office visits, and medications. The model compared CE to SBFT following ileo-colonoscopy and secondarily compared CE to SBFT for initial evaluation.

RESULTS: Aggregate charges for newly diagnosed, medically managed patients are approximately \$8295. Patients requiring aggressive medical management costs are \$29508; requiring hospitalization, \$49074. At sensitivity > 98.7% and specificity of > 86.4%, CE is less costly than SBFT.

CONCLUSION: Costs of CE for diagnostic evaluation

INTRODUCTION

Crohn's disease (CD) is a chronic, transmural inflammatory bowel disease that primarily involves the small bowel. Symptoms of CD are often non-specific and vary among afflicted individuals. Symptoms may include abdominal pain, diarrhea, vomiting, gastrointestinal bleeding, iron deficiency anemia, weight loss, and fevers^[1]. Crohn's disease can also be associated with extra-intestinal manifestations such as skin rashes, arthritis, and uveitis^[2]. While any segment of the gastrointestinal tract can be affected, the small intestine is involved in approximately 70% of patients and up to 30% of patients will have their disease limited solely to the small bowel, particularly the ileum^[2].

There is no single test that establishes the diagnosis of CD. Diagnosis is based on a combination of clinical, endoscopic, radiographic, and laboratory findings. The sequence of diagnostic tests attempting to establish the diagnosis of CD is often based upon the patient's presenting symptoms. For example, when small bowel CD is suspected clinically, ileo-colonoscopy along with small bowel barium radiography [e.g. small bowel follow-through (SBFT)] has traditionally been performed^[3]. However, barium radiographic studies are limited by poor sensitivity for detecting early lesions of CD (e.g. aphthous erosions/

ulcerations) and ileo-colonoscopy evaluation is confined to only the most distal 5-15 cm of the ileum (terminal ileum). In addition, the non-specific symptoms of CD often lead to delays in obtaining a definitive diagnosis and therefore the institution of appropriate and targeted disease therapy^[4-6]. Rath *et al*^[7] found that 38% of CD patients had an interval of more than a year between onset of symptoms and definitive diagnosis.

Because of delays in diagnosing small bowel CD, the costs to payers that are associated with indeterminate tests, repeat endoscopic and radiographic procedures, frequent physician visits, and hospitalizations are likely to be substantial^[8,9]. Thus, there is a need for an efficient and effective algorithm of diagnostic tests leading to a definitive diagnosis of CD. Optimizing such a diagnostic pathway should have substantial benefits for both patients and payers.

Until recently, the small bowel has been the most difficult part of the gastrointestinal tract to evaluate endoscopically. Since being introduced in 2001, small bowel capsule endoscopy (CE) has facilitated easy access for direct investigation of the entire small bowel mucosa and as a result has revolutionized the diagnosis and management of small bowel diseases including obscure gastrointestinal (GI) bleeding, iron deficiency anemia, CD, polyposis syndromes/tumors, and celiac disease. Because of its ability to examine the entire mucosa of the small intestine, capsule endoscopy has the potential for diagnosing suspected CD patients earlier and as a result, direct costs of care may be reduced.

The aim of this study was to derive and evaluate a decision analytic model to determine the economic benefit of CE compared to ileo-colonoscopy and SBFT for the evaluation of suspected CD of the small bowel.

MATERIALS AND METHODS

Model design

We developed a comprehensive model comparing the direct costs of performing CE for the diagnosis and management of suspected CD in a hypothetical cohort of 10 000 patients. Each arm of the model includes the yield of an initial ileo-colonoscopy immediately followed by SBFT or CE based on current clinical practice and guidelines (Figure 1)^[10-16]. If no diagnosis was made after initial ileo-colonoscopy, we assumed that patients would then receive either a SBFT or CE. In a secondary analysis, we eliminated the initial ileo-colonoscopy to determine the relative global costs using SBFT or CE as an initial diagnostic strategy (Figure 2).

In constructing the model to reflect accepted standards of care for suspected CD we used guidance from clinical experts in CD (JAL, IMG) and the 2005 International Conference on Capsule Endoscopy (ICCE) consensus statement^[10-17]. For economic comparators, we utilized the economic analysis of Goldfarb *et al*^[18] concerning evaluation of suspected CD using CE and publications identified by a systematic literature review for guidance in building and populating the model.

The Goldfarb economic analysis compared CE to traditional modalities for the diagnosis of suspected small bowel CD^[18]. Based on published diagnostic yields of 69.6% for CE *vs* 53.9% for a combined approach with ileo-colonoscopy and SBFT, their analysis determined CE to be a less costly strategy as long as its diagnostic yield was 64.1% or greater. The authors also reported CE to be less costly as a primary diagnostic tool in this clinical setting^[18]. These data are provocative, but CE and ileo-colonoscopy/SBFT as mutually exclusive diagnostic strategies may not translate into every day clinical practice. In addition, the included trials were not subjected to a systematic review analysis to evaluate for study heterogeneity.

Model assumptions and definitions

Literature review: To estimate key model variables we performed a systematic literature review to investigate the evidence concerning the use of CE for evaluating suspected CD. The MeSH search terms and strategies employed by third party payers were used for identifying relevant publications. Multiple MEDLINE, EMBASE, COCHRANE TRIALS, and BIOSIS searches were completed using MeSH search terms including “capsule endoscopy”, “Pillcam”, “wireless endoscopy”, “wireless capsule endoscopy”, “video endoscopy”, “video capsule endoscopy”, “M2A capsule endoscopy”, “endoscopy”, “ileum endoscopy”, “ileal endoscopy”, “small bowel imaging”, “small intestine imaging”, “Crohn’s disease”, “Crohn’s disease diagnosis”, “Crohn’s disease radiology”, “Crohn’s disease imaging”, “Crohn’s disease costs”, “Crohn’s disease payments”, “Crohn’s disease reimbursement”.

A total of 891 citations were derived from the published literature searches (January 1970 - January 2008). 792 (89%) were published in English and 141 (16%) were review articles; 29 were published in German, 28 in Spanish, 7 each in Japanese and French, and 5 in Italian. In all cases, we reviewed full manuscripts/reports and case reports with sufficient evaluable information but did not include published abstracts. The literature reporting on ultrasound, magnetic resonance (MR), and computed tomography (CT) enterography in CD continues to grow but remains inconsistent in estimates of diagnostic yield and cost; consequently, we chose not to compare these techniques.

Patients

Using a decision analysis software (TreeAge Pro Suite 2006 Release 0.3, Williamstown, MA), we evaluated a hypothetical cohort of 10 000 patients who had suspected CD as defined by the 2005 ICCE Consensus conference^[17]. Ileo-colonoscopy was negative and was assumed to effectively exclude lesions within the reach of a colonoscope. Therefore, CD lesions modeled were defined to be located proximal to the distal terminal ileum.

This model estimates the total expected global costs to a third party payer of the initial diagnostic work-up as well as expected follow-up costs of managing these patients for one year after diagnosis of CD, including procedure-related adverse events, subsequent hospitalizations, office

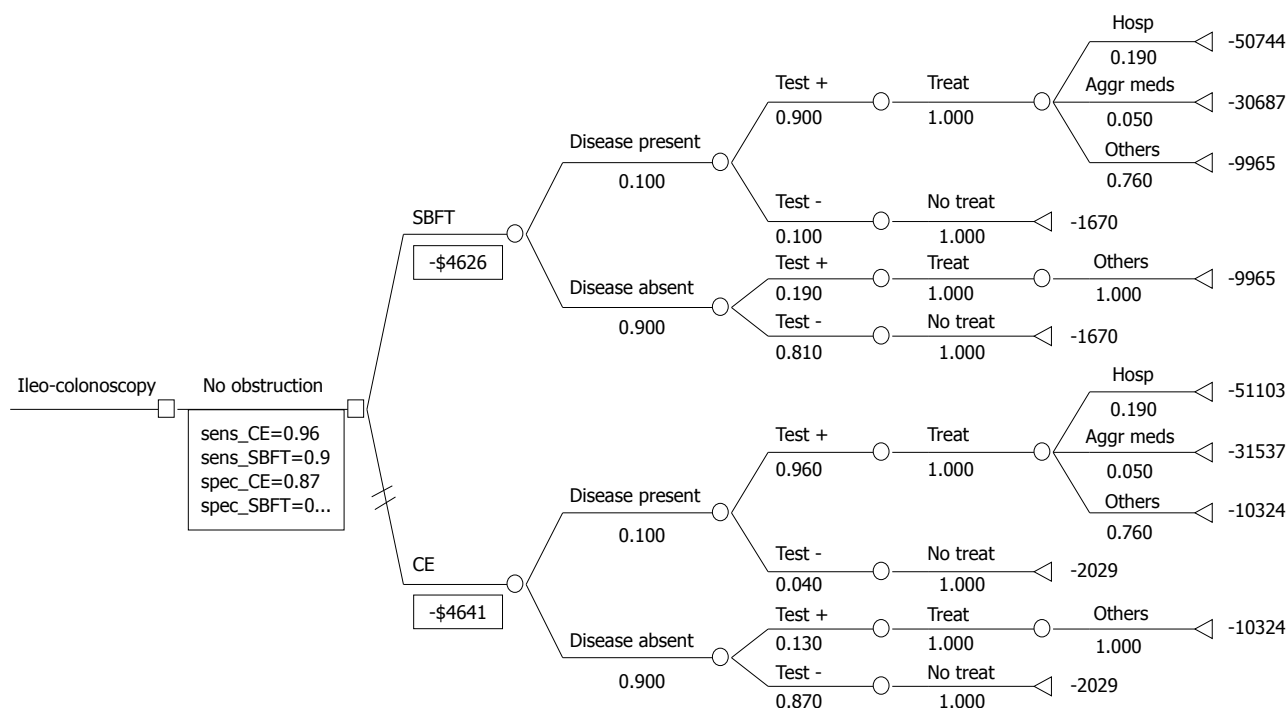


Figure 1 One-year cost model for diagnosis and management of suspected Crohn's disease (CD) of the small bowel with ileo-colonoscopy as first-line diagnostic technique. SBFT: Small bowel follow-through; CE: Capsule endoscopy.

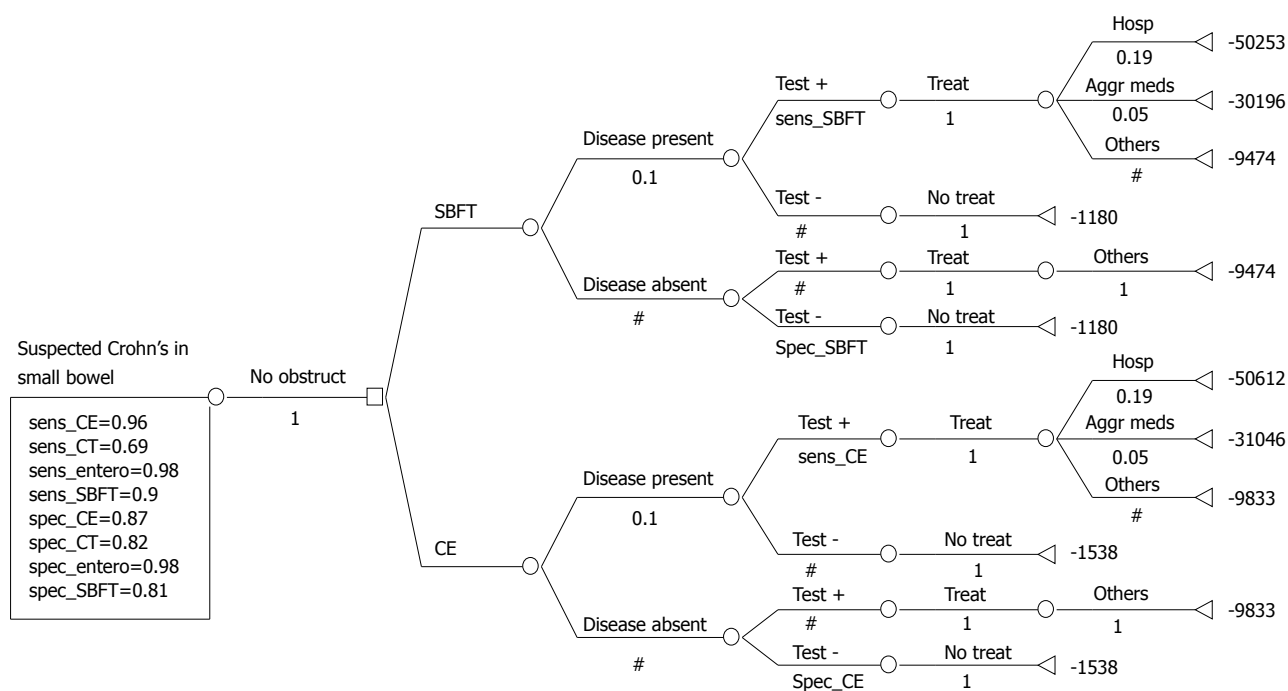


Figure 2 One-year cost model for diagnosis and management of suspected CD of the small bowel with ileo-colonoscopy/SBFT or capsule endoscopy as first-line diagnostic technique. CT: Computed tomography.

visits, laboratory tests, and medications. We chose this design because this economic model allows predictive modeling of the annual economic burden for a chronic disease like CD. Payers are concerned about the rate of medical resource utilization of both inpatient and outpatient services and are not generally interested in other burdens to the patient such as sick time off or other opportunity loss. In addition, because of enrollee turnover

rates in health plans, payers are most interested in models that study short-term (1-2 years) rather than long-term (> 2 years) economic burdens. Finally, payers are interested in imaging and diagnostic tests that are not additive (i.e. leads to treatment change without requiring further testing).

We developed a cost model based on the annual costs of care for diagnosis and management of a patient with suspected CD^[19].

Previously, Feagan *et al*^[19] used medical claims data to identify and stratify patients with CD into three mutually exclusive disease severity groups: Group 1, required an inpatient hospitalization associated with a primary/secondary diagnosis of CD; Group 2, required aggressive pharmacotherapy, defined as chronic glucocorticoid (> 10 mg/d) or immunosuppressive drug (purine antimetabolites/methotrexate) therapy, for > 6 mo and/or anti-TNF monoclonal antibody therapy; or Group 3, all remaining patients (Table 1). As with prior economic evaluations and models reported in the biomedical literature, we used Medicare reimbursement data as surrogates of cost^[18,19]. For tests and procedures, the model was populated with national average allowable 2007 Medicare reimbursement data (Table 2).

We were interested in determining total payer costs associated with the diagnostic evaluation and management of suspected CD. Therefore we evaluated typical hospitalization, outpatient, and medication costs associated with the care of a patient being evaluated for suspected CD of the small bowel. To estimate the added cost of anti-TNF monoclonal antibody therapy (e.g. Remicade[®]), we identified cost-effectiveness analyses on the use of these agents for treatment of aggressive CD. Recent literature has estimated that 25% of patients in this group would receive anti-TNF monoclonal antibody therapy^[20,21]. Based on the reported shifts in utilization, we estimated an annual cost of \$65 000 for this class of drugs and calculated a weighted average cost of \$16 250 per patient to simulate the expected cost of these agents in the aggressive medical management group (Feagan *et al*^[19] Group 2).

Annual costs were then estimated as a weighted average based on the likelihood of the correct diagnosis multiplied by the expected costs of follow-up care by each patient type. We estimated in-patient hospital costs by examining charge data from the Nationwide Inpatient Sample (NIS) which is one in a family of databases and software tools developed as part of the Healthcare Cost and Utilization Project (HCUP), a Federal-State-Industry partnership sponsored by the Agency for Healthcare Research and Quality (Rockville, MD). This database permits searches of in-patient costs according to ICD-9 disease classifications. The database includes historical information and permits comparisons to inflation-adjusted calculations.

The systematic literature review described above was used to identify mean sensitivity and specificity estimates for the comparator diagnostic techniques (Table 3). These values were used to populate the model as were the inflation-adjusted charges described in the manuscript by Feagan *et al*^[19]. In addition, a series of sensitivity analyses, including a Monte Carlo analysis, were performed to determine the relative strengths and weaknesses of utilizing CE relative to other diagnostic testing options in patients with suspected CD. Monte Carlo simulation creates a multi-way sensitivity analysis. The base-case decision tree was repeatedly analyzed using inputs with appropriate distributions to determine the proportion

Table 1 Aggregate charges for each therapeutic grouping used in model

Procedure	Group 1	Group 2	Group 3
Base charge ¹	\$49 074	\$13 258	\$8 295
Monoclonal therapy ²	-	\$16 250	-
Colon/SBFT ³	\$50 253	\$30 687	\$9 474
CE ⁴	\$50 612	\$31 046	\$9 833

¹Groups 1, 2, and 3 base annual charges adjusted for inflation from Feagan *et al* by consumer price index (CPI) to estimate 2007 charges; ²Base annual charge for monoclonal (Infliximab) therapy for Crohn's disease was estimated as \$65 000. We assumed 25% of patients requiring aggressive medical management were treated with this agent and adjusted Group 2 by \$16 250; ³Colon/SBFT procedure charge \$1179; ⁴CE procedure charge \$1538. SBFT: Small bowel follow-through; CE: Capsule endoscopy.

of runs each strategy was identified as the least costly initial strategy. The following variables were used in the simulation: costs and sensitivity of tests and rates of procedural complications. Each simulation performed included 10 000 trials. Adding the potential cost of complication(s) following possible capsule retention did not change the economic results (data not shown).

RESULTS

Figure 2 depicts the derived cost model. All patients with suspected CD are initially examined with ileo-colonoscopy followed by SBFT or CE. Overall, patients evaluated and treated by the CE pathway cost \$4641 whereas those evaluated by SBFT cost \$4626, a difference of \$15 per patient slightly in favor of SBFT. Patients with positive small bowel findings on CE who required hospitalization cost \$51 103 and those requiring aggressive medical management cost \$31 537. These values were comparable to the SBFT pathway. In the secondary analysis, patients managed by CE as the initial diagnostic test cost \$4150 or \$15 per patient more than those initially evaluated with SBFT. These results were comparable to the prior results because of the lower cost of omitting an initial ileo-colonoscopy at an estimated cost of \$491.

We conducted a Monte Carlo simulation involving 10 000 patients undergoing evaluation for suspected CD. In evaluating the results shown in Figure 3, there is evidence that the probability of incurring a charge of at least \$1670 is > 97.5%; there is less than a 2.5% probability of incurring a charge of more than \$9965. The mean cost for evaluation of the patient with suspected small bowel CD is \$4601 ± 7467 (SD) for this 10 000 patient model cohort examined by ileo-colonoscopy first, immediately followed by SBFT. Likewise, there is evidence that the probability of incurring a charge of at least \$2029 is > 97.5%; there is less than a 2.5% probability of incurring a charge of more than \$10 324 in those evaluated by ileo-colonoscopy first, immediately followed by CE. The mean cost for evaluating a patient with suspected CD in that group is \$4604 ± 7563 (SD) for the 10 000 patient simulation. The results of the Monte Carlo simulation are essentially identical (\$4604 for SBFT

Table 2 Procedure charges used for the model according to the 2007 medicare fee schedule

HCPCS code	Procedure	Reimbursement	Colonoscopy w/SBFT	Capsule endoscopy
45380	Colonoscopy, flexible, proximal to splenic flexure; with biopsy, single or multiple	\$491.15	x	x
74249	Radiological examination, gastrointestinal tract, upper, air contrast, with specific high density barium, effervescent agent, with or without glucagon; with small intestine follow-through	\$177.87	x	
88305	Level IV-surgical pathology, gross and microscopic examination	\$115.38	x	
88323	Consultation and report on referred material requiring preparation of slides	\$150.09	x	
91110	Gastrointestinal tract imaging, intraluminal (e.g. capsule endoscopy), esophagus through ileum, with physician interpretation and report	\$950.00		x
99242	Office (gastroenterologist) consultation (Level 2)	\$97.37	x	x
		Additional Medications ¹	\$50	
		Total	\$1179.00	\$1538.00

¹Additional medications: Sedatives, anxiolytics, and anti-emetics administered during a routine procedure.

Table 3 Model inputs

Input	Mean (range)	Ref.
CE sensitivity (sens_CE)	0.96 (0.80-0.99)	[3,14-17,26,27]
CE specificity (spec_CE)	0.87 (0.80-0.99)	[3,14-17,26,27]
SBFT sensitivity (sens_SBFT)	0.96 (0.80-0.99)	[3,14-17,26,27]
SBFT specificity (spec_SBFT)	0.81(0.80-0.99)	[3, 14-17, 26,27]
Disease present/absent	0.10/0.90	[1,2,4]
Hospitalization frequency (Group 1)	0.19	[18]
Aggressive medical management frequency (Group 2)	0.05	[18]
Other management frequency (Group 3)	0.76	[18]

vs \$4601 for CE) for each technique and represent a real-world scenario of relevant costs.

The results of one-way sensitivity analyses on the diagnostic sensitivity and specificity of SBFT and CE are shown in Figure 4 for patients who receive an initial ileo-colonoscopy. The graphs depict how sensitivity (x-axis) and cost (y-axis) (i.e. expected value) interplay. As expected, the cost of performing either test decreases with increasing sensitivity or specificity. The cost of performing SBFT vs CE is essentially equivalent over a wide range of sensitivity values, particularly in the highest ranges of sensitivity and specificity, which might be achieved by an experienced clinician. At a sensitivity > 95.1%, CE becomes less costly than SBFT; at a specificity of > 87.2%, CE is less costly than SBFT. As expected, the results are identical in the secondary analysis (data not shown).

DISCUSSION

Capsule endoscopy is a valuable tool for the study of patients in whom a clinical suspicion of small bowel CD is raised. We found in this present study, applying a conservative costing methodology, incorporating ileo-colonoscopy with SBFT, and CE, demonstrates that CE is marginally less costly overall than use of SBFT despite CE being a slightly more costly test. Secondary analysis demonstrates the cost advantage of CE is robust over a wide range of diagnostic sensitivity and specificity values. Thus, following ileo-colonoscopy, there appears to be

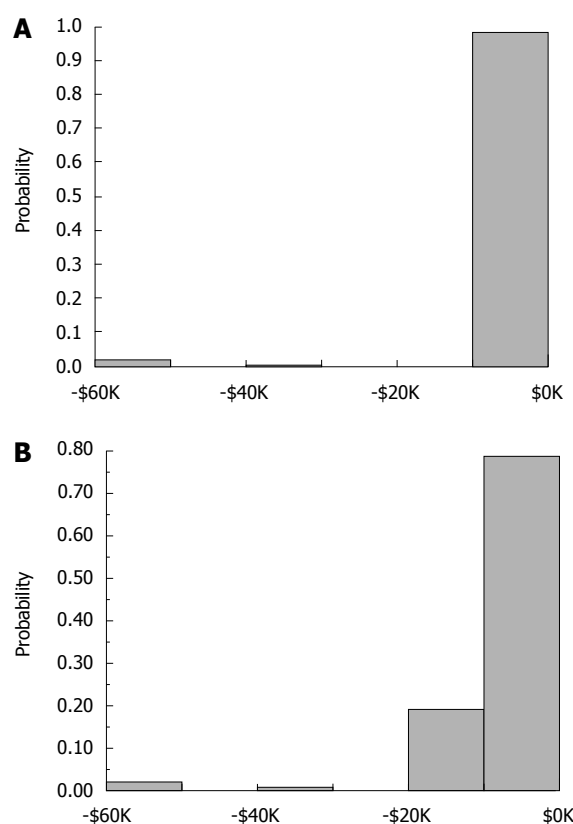


Figure 3 Monte Carlo simulation at ileo-colonoscopy. A: Monte Carlo simulation at SBFT; B: Monte Carlo simulation at CE.

evidence and a cost basis to encourage the use of CE initially (e.g. in place of SBFT) in diagnostic algorithms for suspected small bowel CD.

Suspected CD patients often go months and sometimes years without a definitive diagnosis, especially those with more mild or moderate symptoms and with disease that is isolated to the small bowel. For example, ileo-colonoscopy may be normal in such individuals and a functional bowel disorder may be suspected. Symptoms of CD, especially in the early stages, may mimic functional bowel disease, such as irritable bowel syndrome, confounding the differential diagnosis^[19]. Radiographic diagnostic tests such as SBFT may not be sensitive enough to detect early small bowel mucosal changes (aphthae)

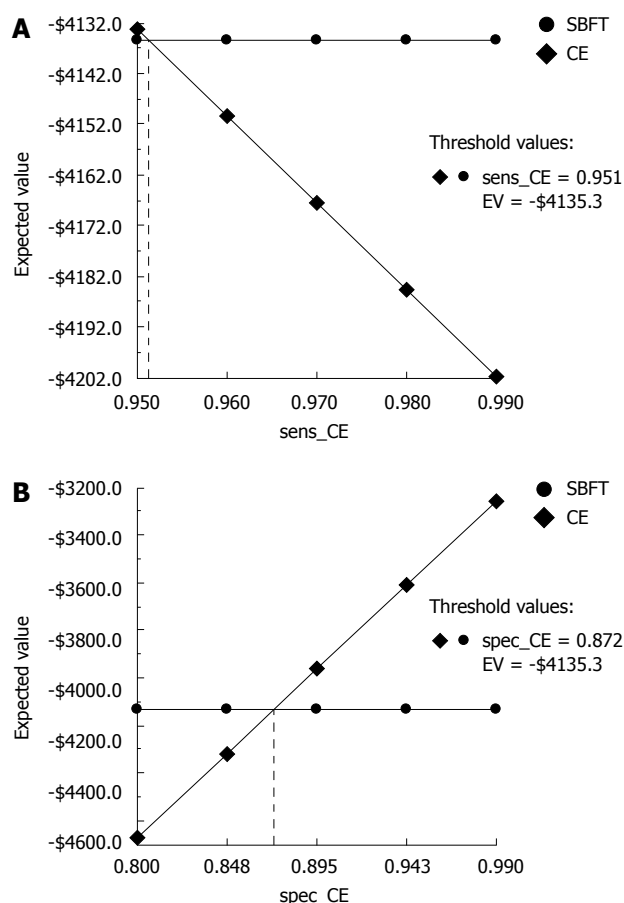


Figure 4 Sensitivity analysis on CE sensitivity and specificity. A: Sensitivity analysis on sens_CE; B: Sensitivity analysis on spec_CE.

and enteroclysis, although a more sensitive test than SBFT is no longer routinely used due to the difficulty of performing the exam, its limited availability, and patient discomfort associated with the procedure. Moreover, exposure to radiation with these tests is a genuine concern among patients and physicians. As a result, these standard radiographic diagnostic testing approaches (SBFT and enteroclysis) can contribute to a delay in diagnosis and hence costs to the payer. While CT enterography may be a superior imaging modality to SBFT and enteroclysis for suspected CD, at present the test has limited clinical availability and as such, SBFT remains the initial study following ileo-colonoscopy in most clinical practices. For those patients with a negative ileo-colonoscopy and negative SBFT in whom obstructive symptoms raise concern for possible capsule retention, CT enterography or the Agile Patency Capsule performed prior to standard small bowel capsule endoscopy may be the next best option to evaluate these patients.

There are a number of limitations to this present study. The model inputs for the test characteristics for diagnostic tests were extracted from the literature based on clinical studies that included selected patients. These patient populations may not reflect the spectrum of patients seen in usual clinical practice. We have attempted however, to temper this limitation by performing sensitivity analyses over a wide range of model estimates,

including a Monte Carlo simulation^[22]. Another limitation of this study is that there are only a limited number of publications that report findings on the use of CE for CD. Moreover, sample sizes in these trials have invariably been small. However, a meta-analysis of available prospective trials comparing CE with one or more alternate diagnostic modalities for the diagnosis of suspected or established CD, found an incremental yield of 43% (95% CI = 29% to 56%) for CE over SBFT for the diagnosis of small bowel CD, with a number needed to test of only 2 patients for an additional positive finding with CE^[23]. An updated meta-analysis showed that the yield favors CE compared to SBFT for suspected CD^[24-26]. Analysis of CE *vs* colonoscopy with ileoscopy (incremental yield with CE = 16%, 95% CI = 3% to 30%) and CT enterography/enteroclysis (incremental yield with CE = 32%, 95% CI = 3% to 61%) also found a significantly improved yield with CE. We utilized data from these meta-analyses to better populate our model estimates.

Our cost-analysis was limited to a third-party payer perspective, although there are additional relevant perspectives. For example, indirect costs to the patient and the cost to the gastroenterologist and their capacity to deliver care (provide CE examination) was not evaluated. In the case of the ability of gastroenterologists to provide care, CE is now a widely accepted diagnostic test that most gastroenterology practices have available and utilize. Moreover, the time horizon for this analysis was limited to one year. However, payers are most interested in models that study short-term (1-2 years) rather than long-term (> 2 years) economic burdens due in part to attrition of members in health plans.

Though CE is a promising technology for the evaluation of patients with CD, there are a number of concerns with its use in this population. The possibility of capsule retention due to known or suspected strictures is a concern in this patient group. The rate of capsule retention in the largest published series of CE for obscure gastrointestinal bleeding was 0.75% (7/934 patients) despite a negative SBFT in 6/7 patients^[27]. All patients underwent surgical resection, and pathology explaining the reason for each patient's symptoms was found at the site of capsule impaction (a so-called therapeutic complication). Due to the stenosing nature of CD, however, a higher rate of capsule retention may be expected if CE is performed in these patients. Rates of capsule retention in patients with CD range from 0% to 13% despite small bowel imaging performed prior to CE in nearly all patients^[28,29]. However, the rate of capsule retention is significantly higher in patients with known CD compared to those with suspected CD.

Perhaps the most significant issue facing CE in the evaluation of CD is the lack of specificity and its inability to provide pathologic confirmation of observed findings. In clinical studies, a variety of findings encompassing a broad range of severity have been described as potentially consistent with the diagnosis of CD, and thereby a positive result with CE. As a diagnosis of CD has far-reaching effects on the psychological and financial health

of patients as well as their physical health, caution in making this diagnosis based on subtle CE findings is appropriate. The long-term clinical significance of finding occasional mucosal breaks in the small bowel on CE is presently unclear, and will require prospective studies to clarify the issue.

In summary, this economic model, along with several clinical trials suggest that CE is a valuable tool in the diagnosis and subsequent management of patients with suspected CD. Data from the studies reviewed in this article suggest improved outcomes after CE due to targeted medical therapy for those patients found to have lesions consistent with CD, as well as a change in therapeutic strategy in those patients with normal capsule examinations^[25,30]. The burden of delay in definitive diagnosis of a debilitating chronic disease is never more apparent than in CD where earlier intervention and treatment makes a significant clinical impact. New technologies and diagnostics, such as CE, require evidence from clinical and payer decision makers who demand rigorous proof of clinical and economic utility. This model provides economic evidence in favor of CE for use immediately following ileo-colonoscopy in the diagnostic pathway for suspected CD.

COMMENTS

Background

Using capsule endoscopy (CE) earlier in diagnosis of suspected Crohn's Disease (CD) may reduce direct costs of care due to ability to examine the entire mucosa of the small intestine; currently, no single test establishes this diagnosis.

Research frontiers

Accurate, early diagnosis of CD is important to patient management decisions. Current therapies may be expensive and their potential inappropriate clinical use due to incorrect diagnosis is troublesome. CE represents a diagnostic technology that may improve overall diagnostic yield therein obviating potentially substantial clinical and economic loss.

Innovations and breakthroughs

CE is an important breakthrough diagnostic technology that is used in a wide number of clinical settings. The present analysis considers its use in suspected small bowel CD. The findings here extend previous cost analyses. The economic model presented here is the first to combine diagnostic and therapeutic costs associated with this condition.

Applications

The results of this study may assist in developing cost-effective diagnostic guidelines for evaluation of suspected CD.

Terminology

Crohn's disease (CD) is a chronic, transmural inflammatory bowel disease that primarily involves the small bowel. Capsule endoscopy (CE) is a technique that directly visualizes the surface of the small bowel where abnormalities due to CD may be identified.

Peer review

This study presents a valuable economic model that combines the cost-effectiveness of diagnosis and treatment of patients with suspected CD. The results demonstrate the value of capsule endoscopy in the overall management of patients with this condition. These results may be useful in future studies of cost-effective approaches to the diagnosis and treatment of CD as well as to considerations of diagnostic selection in clinical guidelines and practice.

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Hepatic steatosis in overweight/obese females: New screening method for those at risk

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Obesity of a severe grade was represented more in the group of IR individuals ($P = 0.01$). Hepatic steatosis, revealed at ultrasound, was more pronounced in IR than in non-IR subjects ($P = 0.005$). The two groups also demonstrated a clear difference in longitudinal spleen diameter and blood pressure, with raised and significant values in the IR group. Metabolic syndrome was frequent in the IR group, and was not modified when adjusted for menopause ($P = 0.001$). At linear regression, the β values of waist circumference and body mass index predicting HOMA were 0.295, $P = 0.007$ and 0.41, $P = 0.0001$, respectively. Measures of spleen longitudinal diameter were well predicted by body mass index (BMI) values, $\beta = 0.35$, $P = 0.01$, and by HOMA, $\beta = 0.41$, $P = 0.0001$. Blood pressure was predicted by HOMA values, $\beta = 0.39$, $P = 0.0001$. HOMA and hepatic steatosis were highly associated ($\rho = 0.34$, $P = 0.002$). Interestingly, IR patients were almost twice as likely to have hepatic steatosis as non-IR patients. Among the MS criteria, blood pressure was very accurate in identifying the presence of IR (AUROC for systolic blood pressure 0.66, cut-off 125 mm of Hg, sensibility 64%, specificity 75%; AUROC for diastolic blood pressure 0.70, cut-off 85 mm of Hg, sensibility 54.5%, specificity 75%).

Abstract

AIM: To identify which parameters could help to distinguish the "metabolically benign obesity", which is not accompanied by insulin resistance (IR) and early atherosclerosis.

METHODS: Eighty two of 124 overweight/obese females formed the study population, which was divided into two groups (52 and 30 subjects, respectively) with and without IR according to a HO meostatic Metabolic Assessment (HOMA) cut-off of 2, and were studied in a cross-sectional manner. The main outcome measures were waist circumference, serum uric acid, high-density lipoprotein-cholesterol and triglycerides, alanine amino-transferase, blood pressure and the two imaging parameters, hepatic steatosis and longitudinal diameter of the spleen, which were measured in relation to the presence/absence of IR.

RESULTS: A variable grade of visceral obesity was observed in all subjects with the exception of three.

CONCLUSION: As health care costs are skyrocketing, reliable and mainly inexpensive tools are advisable to better define subjects who really need to lose weight.

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Key words: Body mass index; Cardiovascular disease; Fatty liver; Insulin resistance; Metabolic fitness; Obesity

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INTRODUCTION

The incidence of both Overweight/Obesity (O/O) and Metabolic Syndrome (MS) is dramatically increasing. The generally accepted view is that being overweight causes similar health problems to that of obesity but to a lesser degree. The risk of death from all causes increases throughout the range of moderate and severe increases in body weight for both men and women in all age groups^[1]. Further co-morbidities^[2-4] are more frequent in O/O than in normal-weight females. Unless reversed, this trend predicts an epidemic of cardiovascular disease (CVD). Interventions are required to empower adults to increase physical activity and to modify eating habits. Nevertheless, it is mandatory to establish exactly which individuals should be treated and when. In fact, although the negative health consequences of O/O in the general population are well supported by the available published evidence, health outcomes in certain subgroups seem to be improved at an increased body mass index (BMI), a phenomenon known as the obesity survival paradox^[5]. Furthermore, there is a high prevalence of clustering of cardiometabolic abnormalities among normal-weight individuals and a high prevalence of O/O individuals who are metabolically healthy^[6]. Consequently, “metabolically benign obesity” which is not accompanied by insulin resistance (IR) and early atherosclerosis has recently been postulated to exist in humans^[7].

A way of getting to the grass-roots of the problem could be to tackle the mechanisms underlying O/O. Evidence that this phenomenon can be regarded as a low-grade chronic inflammatory state comes from numerous studies^[8]. Moreover, inflammation links obesity to early tumor promotion^[9]. Inflammatory disease could represent a compensatory mechanism for increased adipose tissue turnover^[10]. Apart from diabetes and atherosclerosis, another associated high-risk condition is non-alcoholic fatty liver disease (NAFLD), the etiologic factors of which include IR^[11], high levels of serum interleukin-6 (IL-6)^[12] and mitochondrial dysfunction^[13]. The link between NAFLD and CVD has recently been established by the fact that the liver is involved in regulating/secreting numerous CVD risk factors^[14], notably a cytokine [tumor necrosis factor- α (TNF- α)], an acute-phase protein [C-reactive protein (CRP)], glucose, lipoproteins, coagulation factors (plasminogen activator inhibitor-1^[15]) and a substance which increases blood pressure (angiotensin II^[16]).

The NAFLD rate in the general adult population is increasing with large numbers, i.e. 20%^[17], in obese non-diabetic adults being much higher^[18]. Obviously, a clear differentiation between “simple” fatty liver (FL) and non-alcoholic steatohepatitis (NASH) in the spectrum of NAFLD is quite impossible without liver biopsy which is not always performed for ethical and technical issues. For this reason, we generally speak of hepatic steatosis (HS). Ultrasound (US) is widely used to detect HS, without exposing subjects to ionizing radiations. Indeed, more specific imaging techniques^[19] are costly and not always useful in close follow-up. Tsushima *et al*^[20] first identified the so-called liver-spleen axis in NAFLD patients. It has recently been proposed that increased spleen volume and

serum levels of high sensibility (hs)-CRP contextually with HS, which “*per se*” unravels an inflammatory status^[14], characterize young adult obese subjects^[21]. Up-to-date findings from a nationally representative sample of adults indicate that the prevalence of MS increases substantially with increasing levels of serum uric acid (UA)^[22]. Actually, the menopause is associated with the aggravation of total cholesterol, LDL cholesterol and apolipoprotein B^[23], representing a physiologic event which should be taken into consideration when evaluating multiple cardiovascular risk factors in O/O females, even though this status is highly correlated with age. Finally, researchers have demonstrated that elevated alanine aminotransferase (ALT) levels are independently associated with increased CVD- or diabetes-related mortality^[24].

With numerous studies showing that O/O is a disease requiring long-term commitment to achieve the desired results, and that these results should be achieved as soon as possible^[25], we aimed to identify which parameters could help distinguish between healthy and non healthy O/O individuals, by individualizing the boundaries within which is possible to “watch and wait”. As health care costs are skyrocketing, the key point is to utilize tools which are reliable and mainly inexpensive. To achieve this aim we analyzed an anthropometric index, i.e. waist circumference (WC), in addition to the metabolic parameters, serum UA, high-density lipoprotein-cholesterol (HDL) and triglycerides, liver enzyme activity i.e. ALT, a CVD index, i.e. blood pressure, and two imaging parameters which were HS and spleen size, in relation to the presence/absence of IR.

MATERIALS AND METHODS

This research was performed by screening 124 consecutive female subjects with established (at least 4 years) O/O from February 2008 to April 2009. The study was carried out according to the principles of the Declaration of Helsinki and an informed written consent was obtained from each patient.

Exclusion criteria

Of the 124 initial subjects, 11 patients, who had undergone steroid therapy (four with bronchial asthma, five with rheumatoid arthritis and two with neuritis), as well as 15, who had received one or more of the following drugs, i.e. aspirin, metformin, statins and fibrates, were excluded from the study because these prior treatments may have caused a change in laboratory data. Three persons were excluded due to the detection of marked intestinal meteorism which made it difficult to perform US. Eight others were excluded due to the presence of co-morbidity (chronic viral hepatitis, alcohol abuse), and five subjects were considered drop-outs, because they avoided undergoing full laboratory-instrumental examinations.

Inclusion criteria

The remaining 82 subjects whose median age was 46.5 (14-64) years, were divided into two groups on the basis of the presence or absence of IR, and formed the study

population. The degree of overweight or obesity was established on the basis of BMI cut-off points of 25-29.9, 30-34.9, 35-39.9 and $> 40 \text{ kg/m}^2$, respectively. Visceral obesity was identified by measuring WC at the midpoint between the lower border of the rib cage and the iliac crest. MS was defined according to the revised Adults Treatment Panel III (2001), and three or more criteria were considered: plasma glucose concentration of at least 100 mg/dL, WC $> 88 \text{ cm}$, serum HDL concentration $< 50 \text{ mg/dL}$, blood pressure of at least 130/85 mmHg, and serum triglyceride concentration of at least 150 mg/dL. HS was associated with recent US features of "bright liver", with or without aminotransferase increase of unknown origin, in the absence of other chronic liver disease. Women were classified as being menopausal if they had not menstruated in the 12 months before examination. IR status was determined by the HOMeostatic Metabolic Assessment (HOMA), which was assessed using the formula: fasting insulin ($\mu\text{U/mL}$) $\times$ fasting glucose (mg/dL)/405^[26]. As a stringent measure of IR, a value of HOMA > 2 was introduced.

Ultrasound evaluation

Sonographic measurements were performed using an ESAOTE *Technos* (Genoa, Italy). Briefly, spleen longitudinal diameter (SLD) was measured by postero-lateral scanning. The maximum length and the cranio-caudal were measured and then averaged. All the indices were measured thrice directly from the frozen images using an electronic caliper. The classification of "bright liver" or HS was based on the following scale of hyperechogenicity: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney^[27].

Blood pressure measurements

The systolic/diastolic blood pressure (SBP, DBP) was the average of three consecutive readings taken by the physician during the day, during usual practice hours, and after subjects had rested for five minutes in the sitting position.

Laboratory data

Serum triglycerides, HDL, basal insulin, ALT, and UA were measured using standard in-house procedures. Hs-CRP was determined using an ELISA Kit, human CRP by BioSupply, UK with a sensibility of 0.03 mg/L and a range of normal values of 0.25-1.5 mg/L.

Statistical analysis

HDL data, derived from a normally distributed population [Shapiro-Wilk test (S-W), $P = 0.481$], are expressed as mean plus SD. Variables not normally distributed, such as age (S-W, $P = 0.000$), BMI (S-W, $P = 0.005$), SLD (S-W, $P = 0.002$), WC (S-W, $P = 0.002$), HOMA (S-W, $P = 0.001$), DBP (S-W, $P = 0.001$), SBP (S-W, $P = 0.001$), triglycerides (S-W, $P = 0.003$), UA (S-W, $P = 0.001$), hs-CRP (S-W, $P = 0.001$), and ALT (S-W, $P = 0.001$), are expressed as median (range). HS grades were considered ordinals and managed in the same way. The difference

in medians was assessed by the Mann-Whitney test for independent samples. The difference in means of HDL was evaluated by the Two-Sample t test. The Two-Way Tables cross-tabulated one categorical row variable with one categorical column variable and the significance was set by the Pearson χ^2 test. Frequencies with a small number of the expected values were evaluated by Fisher's exact test. When cross-tabulation was stratified for another dichotomous variable, the Mantel-Haenszel χ^2 test was carried out. Tracking the degree of association between single parameters, i.e. HS scores and triglycerides levels, Spearman's rho for non uniform intervals was used. The Pearson's coefficient (r) was employed to analyze the correlation between HOMA and HDL. When confronted with the question of how accurate a parameter was in identifying the presence of IR, the discrimination with relative cut-off was evaluated using receiver operating characteristic curve (ROC) analysis, graphically expressed as area under the ROC (AUROC). Sensitivity (true positive rate) and specificity (true negative rate) were also weighted for the same purpose. Optimal cut-off was considered the threshold value with the best specificity/sensitivity. To predict the presence of IR, the logistic regression (Enter Method), with relative odds ratios and 95% confidence intervals (CI), was employed utilizing as independent variables, US values for SLD and HS, BMI and WC measurements, blood pressure determinations and UA, HDL, and triglycerides data. To assess the independent effect of a quantitative variable, i.e. HOMA, on the prediction of spleen size (SLD) values and SBP/DBP determinations, linear regression analysis (least squares) was used, evaluating the standardized coefficient beta (β). The same tool was used to verify the connection between IR severity and anthropometric measures. Factor analysis was applied to detect the structure in the relationships among variables selecting a subset of variables, which have the highest correlations with the principal component factors. The Cattell's Scree plot, with relative eigenvalues, was performed to screen the real factors. Extraction of the main components amounted to a variance maximizing (varimax) rotation of the original variable space. The critical value was calculated by the formula: doubling the Pearson's correlation coefficient for 1% level of significance (5.152)/square root of subjects minus 2, i.e. 0.576. The concordance correlation coefficient (ρ_c), which measures precision and accuracy, was adopted to evaluate the degree of pair observations at US. Statistical analysis was performed operating on Systat 12 (Richmond, CA, USA) and MedCalc Version 10.4.8® (Frank Schoonjans) software packages.

RESULTS

In order to allow readers to gauge the internal validity of this study, we determined the minimum required sample size, with a type 1 error of 0.01 and type 2 error of 0.01 when dividing the population in two groups using HOMA (difference of means = 1.8, SD of non-IR females = 0.4 and SD of IR females = 1.8), which was calculated in 26 subjects. In addition, to assess how well

Table 1 Anthropometric, clinical, laboratory and ultrasound data of the study population

Variables [females (82)]	Median (Range) mean $\pm$ SD		P-value
	IR present (52)	IR absent (30)	
BMI (kg/m ²)	33 (25-53.7)	32 (25.6-39.7)	0.78
Overweight (n)	7	6	0.6
Obesity 1 st grade (n)	23	15	0.8
Obesity 2 nd (n)	12	9	0.7
Obesity 3 rd (n)	10	0	0.01 ^a
HS score at US	1 (0-3)	2 (0-3)	0.005
DBP (mmHg)	90 (65-110)	80 (60-110)	0.03
SBP (mmHg)	120 (100-170)	135 (110-180)	0.0008
Triglycerides (mg/dL)	141 (25-386)	113 (41-249)	0.11
HDL (mg/dL)	48.2 $\pm$ 8.6	50.1 $\pm$ 10.1	0.36
Uric acid (mg/dL)	4.3 (2.7-8.2)	4.4 (2.7-7.6)	0.6
ALT (U/L)	22 (11-117)	22 (12-53)	0.9
hs-CRP (mg/L)	2.4 (0.2-11.7)	1.6 (0.3-6.2)	0.71
SLD at US (mm)	115 (92-150)	102 (90-127)	0.0001
MS (n)	18	6	0.0001
Menopause (n)	20	11	0.9

^aFisher's exact test. IR: Insulin resistance; BMI: Body mass index; HS: Hepatic steatosis at US; US: Ultrasonography; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; HDL: High-density lipoprotein-cholesterol; ALT: Alanine aminotransferase; hs-CRP: High sensibility C-reactive protein; SLD: Spleen longitudinal diameter at US; MS: Metabolic syndrome; n: No. of subjects.

the study findings applied to the patients (external validity) the eighty two subjects divided into two cohorts of 52 and 30, were well balanced for BMI ($P = 0.78$), WC ($P = 0.45$) and age ($P = 0.79$), but different for HOMA ($P < 0.001$), and were studied in a cross-sectional manner.

Prevalence

A variable grade of visceral obesity was determined in all subjects with the exception of three (two without IR). Only obesity of the severest grade was represented more in the patients with IR than in the non-IR patients ($P = 0.01$). HS, revealed at US, was more pronounced in subjects with IR than in those without IR ($P = 0.005$). MS was more frequent in IR patients than in non-IR patients. These figures were not modified when adjusted for menopause (Mantel-Haenszel χ^2 test, $P = 0.001$). The same physiological status was unable to distinguish females with IR from those without IR. No difference in ALT activity was detected between the two cohorts on the basis of IR. The two groups demonstrated no resemblance when analyzing SLD, HS and blood pressure, with raised and significant values in the IR group (Table 1).

Association & prediction

Anthropometric measurements in the study females showed a similar pattern of reliably predicting IR severity. In fact, the β values of WC and BMI in predicting HOMA were 0.295, $P = 0.007$ and 0.41, $P = 0.0001$, respectively. HDL levels were not associated with HOMA values ($r = 0.8$, $P = 0.5$). SLD measures were well predicted by BMI values, $\beta = 0.35$, $P = 0.01$, and by HOMA, $\beta = 0.41$, $P = 0.0001$ (Figure 1). SBP determinations were predicted by HOMA values, $\beta = 0.39$, $P = 0.0001$ (Figure 2). HOMA

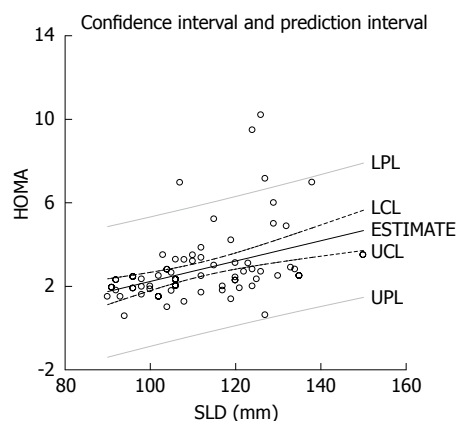


Figure 1 Prediction of spleen size by insulin resistance severity. SLD: Spleen longitudinal diameter at ultrasonography (US); HOMA: HOMEostatic metabolic assessment for insulin resistance; LPL: Lower prediction limit; LCL: Lower confidence limit; UCL: Upper confidence limit; UPL: Upper prediction limit.

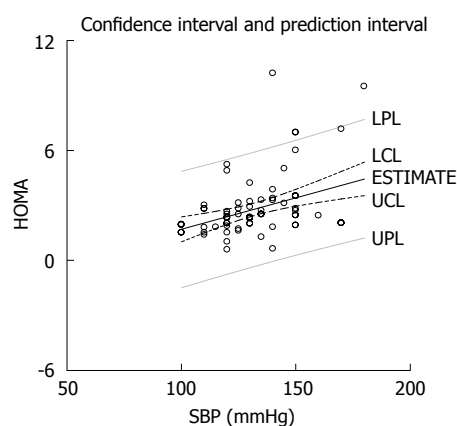


Figure 2 Prediction of systolic blood pressure by insulin resistance severity. SBP: Systolic blood pressure.

and HS scores at US were highly associated ($\rho = 0.34$, $P = 0.002$). In IR patients, the grade of HS severity was not correlated with serum triglycerides levels ($\rho = 0.245$, $P = 0.08$). IR patients were almost twice as likely to have HS as those who did not have IR. Further predictions are shown in Table 2.

The complex of WC, HOMA and SLD as well as age, HS and SBP had the highest correlations among the variables, contributing to the loading of the principal component factors (factor 1 and 2). Triglyceride concentrations (loading factor 3) seemed to play an isolated role (Figure 3).

Accuracy

Among the MS criteria, blood pressure was very accurate in identifying the presence of IR (AUROC for SBP 0.66, cut-off 125 mm of Hg, sensibility 64%, specificity 75%; AUROC for DBP 0.70, cut-off 85 mm of Hg, sensibility 54.5%, specificity 75%).

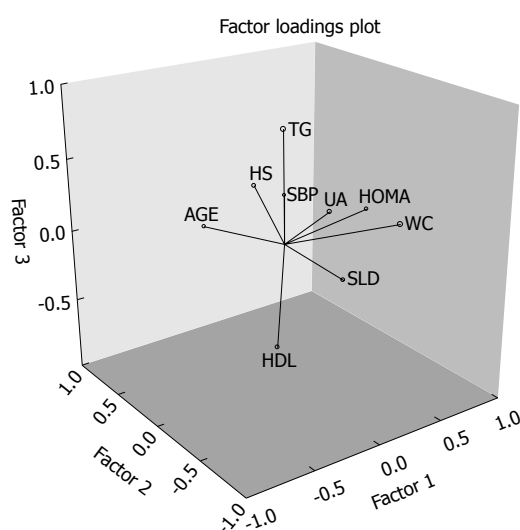
Precision

The concordance correlation coefficient to evaluate the degree of intra-operator pair observations at US was high, $\rho_c = 0.91$.

Table 2 Estimates in predicting insulin resistance

Parameter	OR	Lower	Upper	P-value
SLD	1.073	1.027	1.121	0.002
SBP	1.045	1.015	1.075	0.003
DBP	1.046	1.003	1.090	0.036
HDL	1.022	0.974	1.072	0.378
TG	1.006	0.998	1.014	0.143
UA	1.047	0.676	1.623	0.837
BMI	1.083	0.989	1.187	0.086
WC	1.029	0.991	1.069	0.131
HS	1.971	1.234	3.147	0.004

TG: Triglycerides; UA: Uric acid; WC: Waist circumference.



Factor	1	2	3
WC	0.603	-0.424	0.132
AGE	-0.096	0.810	-0.120
HOMA	0.817	0.163	0.005
HDL	0.029	0.118	-0.804
TG	0.163	0.236	0.679
UA	0.440	0.096	0.091
SBP	0.488	0.669	0.026
HS	0.168	0.580	0.192
SLD	0.594	0.126	-0.461

Figure 3 Unravelling the hidden correlations. Total variance explained by factor: 1 = 20.9%; 2 = 19.36%; WC: Waist circumference; HOMA: HOmeostatic metabolic assessment for insulin resistance; HDL: High-density lipoprotein-cholesterol; TG: Triglycerides; UA: Uric acid; SBP: Systolic blood pressure; HS: Hepatic steatosis at US; SLD: Spleen longitudinal diameter at ultrasonography (US).

DISCUSSION

Authorities claim that (1) every subject with a body weight above the established criteria should be treated, stressing this point even to the extent of suggesting that just brief physician-nutritionist support by telephone could be useful in a busy primary care office^[28], (2) substantial numbers of overweight/obese individuals remain insulin-sensitive^[29]. Consequently, the question we pose is: beyond IR, might some other indicators play a role in identifying novel targets, i.e. typology of O/O patients and timing of interventions^[30]? Accordingly, we propose parameters which may be worth investigating when dealing with the phenotypically O/O and when it is neces-

sary to decide who the metabolically normal subjects are.

The body of pertinent knowledge based on data from the Third National Health and Nutrition Examination Survey testifies that each of the five components of MS, with the possible exception of obesity, independently predicts CVD^[31]. In contrast, abdominal obesity, measured by WC, is a more accurate predictor of CVD risk than general obesity measured by BMI^[32], even though, for a given BMI, women have more subcutaneous adipose tissue and insulin-sensitivity than men^[33]. However, controversy exists over the type of blood pressure increase. Previous results showed that SBP represents the most prevalent type in obese women^[34], being a stronger predictor of CVD than DBP^[35]. Indeed, these data are challenged by more recent research based on longitudinal studies^[36]. In addition, according to some other investigators, both blood pressure and plasma aldosterone correlate better with IR in men than in women^[37], whereas, there is widespread agreement that NAFLD predicts CVD^[38].

In the present study, it can be seen that our data in O/O females supports an association only between the more severe forms of adiposity and the highest grades of IR. Accordingly, MS was characterized by different values in the two groups with and without IR, and HOMA was correlated with severity of HS and to spleen size. The HS score was higher in IR patients, as was another index (hs-CRP), suggesting a possible link between inflammatory status and IR. These findings are consistent with the concept that a partially “benign” obesity, characterized by the absence of IR, lack of HS, or at most the presence of a “light” form of HS, low blood pressure, low expression of a marker of inflammation (SLD) exist in this particular population, at least during the course of this study.

When discussing possible mechanisms for our findings, we pinpoint the following with regard to the liver-spleen axis: (1) peripheral IR determines increased hepatic synthesis of free fatty acids (FFAs) and decreased synthesis of apolipoprotein B, both leading to HS; (2) TNF- α , through JNK-dependent IRS-1 Ser307 phosphorylation, contributes to IR and is responsible for the induction of IL-6 in the liver, both of which aggravate triglyceride accumulation; (3) the overproduction of IL-6, which has “*per se*” a source in visceral and subcutaneous fat, could represent the cause of spleen enlargement in patients with HS *via* uncoupling protein 2^[39]; (4) a further origin of the increased splenic volume may be found in the accumulation of activated macrophages inside this organ *via* monocyte chemoattractant protein-1 which is over-expressed in adipose tissue^[40]; (5) once infiltrated, these macrophages produce a large amount of TNF- α which increases the release of FFAs, resulting in HS. With regard to blood pressure, O/O subjects not only exhibit impaired endothelial function in small arteries but also increased arterial stiffness^[41]. However, IR is associated with greater cardiac reactivity in young women^[42]. Finally, a recent study performed on a large population showed that patients with HS have increased intima media thickness (IMT^[43]).

The limitations and strengths of our study deserve

comment. Concerning the limitations, to verify CVD risk, we should have studied a US parameter marker of early atherosclerosis, e.g. IMT in our population, however, this index, as previously emphasized^[43], is correlated with HS grade. A further drawback may have been the lack of liver biopsies to assess the severity of NAFLD, even though the canonical difference between FL and NASH has been challenged recently^[44]. Finally, we did not determine serum levels of folate which are related to elevated homocysteine or blood pressure. In relation to blood pressure, our data showed a new emerging trend, i.e. rather than focusing primarily on body weight, WC, or BMI as a health indicator, a number of scientists have suggested the use of a measure called Metabolic Fitness (MF). Authors define MF as “the absence of biochemical risk factors associated with obesity or elevated blood pressure”. These measures are much better indicators of chronic disease risk, and reductions in these risks factors are not always dependent on weight loss^[45].

Most people, who are unhappy with their weight, try to lose weight by changing unhealthy practices. This attitude could result in a dramatic increase in unrealistic goals. On the other hand, some lines of research support the notion that a lifestyle-modification program can reduce the risk of O/O-related comorbid conditions despite minimal or no weight loss. What is more, studies investigating weight loss have methodological limitations that restrict the applicability of findings to O/O patients assessed in clinical practice^[46]. When we add all this evidence up, it does raise the question of what will our strategy in the near-to-medium term look like. This debate is still ongoing and is mainly related to health-related quality of life^[47] and the not always favorable balance between costs and treatment outcomes.

COMMENTS

Background

The incidence of Overweight/Obesity in the last few years has dramatically increased in highly developed countries. There has also been a simultaneous rise in the frequency of metabolic syndrome. Being overweight causes similar health problems to that of obesity but to a lesser degree. The risk of death from all causes increases throughout the range of moderate and severe increases in body weight for both men and women in all age groups. Unless reversed, this trend predicts an epidemic of cardiovascular disease.

Research frontiers

It has recently been postulated that “metabolically benign obesity” exists, and is not accompanied by insulin resistance and early atherosclerosis. We sought to identify which parameters could distinguish between healthy and non healthy overweight/obese individuals.

Innovations and breakthroughs

We claim that “hepatocytes are the last cells to be involved in the progressive chain of fat accumulation and probably the first cells to tell us that something is wrong”. If our proposed parameters are to be adopted more widely, they must be validated in a range of settings and different populations; until then, the most important message is that researchers should identify tools that suit clinicians in their clinical setting, allowing them to screen appropriate cases, in order to improve identification of patients who need to lose weight.

Applications

Interventions at home, in the office, and in the community are required to empower adults to increase physical activity and to modify eating habits. Nevertheless, before these intervention strategies are set up, it is mandatory to establish exactly which individuals should be treated and when.

Terminology

The main outcome measures in this study are extremely common, i.e. waist circumference, serum uric acid, high-density lipoprotein-cholesterol and triglycerides, alanine aminotransferase, blood pressure and the two imaging parameters, hepatic steatosis and spleen longitudinal diameter.

Peer review

This is quite an interesting article. The manuscript reports the studies on the use of simpler parameters in assessing the need for medical intervention with respect to healthy and non healthy overweight/obese individuals. It is suggested that adoption of simpler to perform measurements could not only reduce the cost of medical care but also provide more reliable identification of patients in need of weight loss.

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BRIEF ARTICLE

Outcome of nonerosive gastro-esophageal reflux disease patients with pathological acid exposure

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Abstract

AIM: To assess the management and outcome of nonerosive gastro-esophageal reflux disease (NERD) patients who were identified retrospectively, after a 5-year follow-up.

METHODS: We included patients with gastro-esophageal reflux disease (GERD) symptoms who had a negative endoscopy result and pathological 24-h esophageal pH-monitoring while off therapy. We interviewed them after an average period of 5 years (range 3.5-7 years) by means of a structured questionnaire to assess presence of GERD symptoms, related therapy, updated endoscopic data and other features. We assessed predictors of esophagitis development by means of univariate and multivariate statistical analysis.

RESULTS: 260 patients (137 women) were included. Predominant GERD symptoms were heartburn and

regurgitation in 103/260 (40%). 70% received a maintenance treatment, which was proton pump inhibitor (PPI) in 55% of cases. An average number of 1.5 symptomatic relapses per patient/year of follow-up were observed. A progression to erosive gastro-esophageal reflux disease (ERD) was found in 58/193 (30.0%) of patients undergoing repeat endoscopy; 72% of these were Los Angeles grade A-B.

CONCLUSION: This study shows that progression to ERD occurs in about 5% of NERD cases per year, despite therapy. Only two factors consistently and independently influence progression: smoking and absence of PPI therapy.

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Key words: Esophagus; Nonerosive gastro-esophageal reflux disease; Gastro-esophageal reflux disease; Epidemiology; Acidity

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INTRODUCTION

Evaluating the natural history of gastro-esophageal reflux disease (GERD) is useful for a number of reasons, as this knowledge may help to: (1) discern the percentage of the population that will progress from non-erosive to erosive disease with its complications, such as stricture, Barrett's oesophagus, and esophageal adenocarcinoma, or from exclusively esophageal to extraesophageal manifestations; (2) define, assess and validate predictivity of risk factors for such disease complications; (3) determine if medical, surgical or endoscopic therapies are able to positively modify the

natural course of the disease; and (4) determine the need for maintenance therapy to prevent complications and persistent symptoms.

Others^[1,2] have pointed out that many factors make it difficult to study the natural history of GERD, notably the evolving definition of the disease and the lack of a diagnostic standard. As a consequence, few studies in the literature have addressed the issue of defining the natural history of erosive GERD, and even less that of nonerosive gastro-esophageal reflux disease (NERD) or extraesophageal GERD and complications.

Until recently, patients with endoscopic-negative reflux disease were considered to suffer from a milder disease^[3], i.e. requiring less intensive/prolonged treatment and possibly characterized by a better long-term prognosis. This concept was subsequently proven to be incorrect; as an example, the impairment in disease-related quality of life appears to be similar in GERD patients with or without endoscopic esophagitis and is related in both instances to symptom severity^[4]. Also, the symptomatic acute response to proton pump inhibitor (PPI) drugs in patients with or without endoscopic mucosal damage may be worse in NERD^[5,6]. Finally, after discontinuation of acute treatment, symptomatic relapse within 6 mo appears to affect a similarly high proportion of both GERD groups^[7].

Up till now, however, it has not been entirely clear whether and in what proportion GERD patients with symptomatic syndrome but without mucosal damage^[8] may progress to disease with mucosal damage.

The aim of this study was to assess the outcome of NERD patients with pathological acid reflux at entry after a 5-year follow-up, in three Italian tertiary care centres. Additionally, we investigated which factors are related to development of esophagitis.

MATERIALS AND METHODS

Subjects

We retrospectively identified all patients referred to our outpatient departments during the period 1996-2003 for investigation of gastroesophageal (GER) disease symptoms. Subjects were included in the study provided that (1) they were off PPI therapy for at least the preceding 7 d, (2) had a negative upper gastrointestinal (GI) endoscopy, (3) had a 24-h esophageal pH-monitoring result showing pathological GER, i.e. a 24-h esophageal acid exposure greater than 4.4%, corresponding to the 95th percentile of normal Italian subjects and (4) were able to accept a telephone interview^[9]. We interviewed the subjects after an average period of 5 years (range 3.5-7 years) by means of a structured questionnaire, already used in a previous study^[10], to assess presence and type of GERD symptoms (i.e. typical: heartburn or regurgitation, and atypical, i.e. chronic cough, pharyngodynia, hoarseness, chest pain), antireflux drug therapy (presence and type), updated endoscopic data and other features regarding their present clinical status. The questionnaire allowed the patient to identify the most

Table 1 Clinical and demographic characteristics of patient population (mean $\pm$ SD)

M/F	121/139
Age (yr)	49.5 $\pm$ 14
BMI	25.24 $\pm$ 3.7
EAE (%)	
24 h	6.8 $\pm$ 5.4
Upright	5.6 $\pm$ 6.6
Supine	6.8 $\pm$ 7.6
No. of smokers	132
Patients with predominant typical symptoms	103 (39.6%)
Patients with predominant atypical symptoms	142 (54.6%)
Patients with both typical & atypical predominant symptoms	15 (5.7%)

EAE: Esophageal acid exposure; BMI: Body mass index.

bothersome GERD symptom, be it typical or atypical, as the predominant one. Collected data at study entry included demographic characteristics, such as age, gender and body mass index (BMI), the reason for undergoing the examination, pattern of GERD symptoms (Table 1), and the results of index upper GI endoscopy and 24-h esophageal pH-metry. Both investigations were carried out while off PPI therapy.

Statistical analysis

Descriptive statistics consisted of *t* tests for continuous variables and χ^2 tests for categorical variables. We conducted a univariate logistic regression to evaluate the influence of each risk factor, such as gender, age, BMI, smoking, typical and atypical symptoms, use of therapy, pH-monitoring reflux parameters, on the dependent variable, i.e. development of esophagitis. Significant prognostic factors were then subjected to a multivariate analysis with logistic regression to evaluate the association among the determinants while simultaneously controlling for the effect of other variables. We controlled for several covariates, and only variables with *P* > 0.1 were kept in the model. Statistical significance was defined by a two-sided alpha level of 0.05. A Cox model was ultimately used to construct the survival curves.

All analyses were performed using statistical software (SPSS, Chicago, Illinois, USA).

RESULTS

We were able to identify 995 patients overall who were referred for GERD symptoms. 260 patients (137 women) satisfied the inclusion criteria and were included in the study. Mean age at the time of initial evaluation was 50 $\pm$ 14 (SD) years, with a BMI of 25.24 $\pm$ 3.72; 50% were smokers. The clinical and demographic characteristics of patients are listed in Table 1.

Predominant GERD symptoms were typical in 103/260 (40%), atypical in 142/260 (54%) and mixed, i.e. similarly dominating the clinical picture, in the remaining 15/260 (6%) (Figure 1A). The mean percentage time with pH < 4 was 7.1% $\pm$ 2.6%. At interview, after a median follow-up time of 5 years, typical symptoms

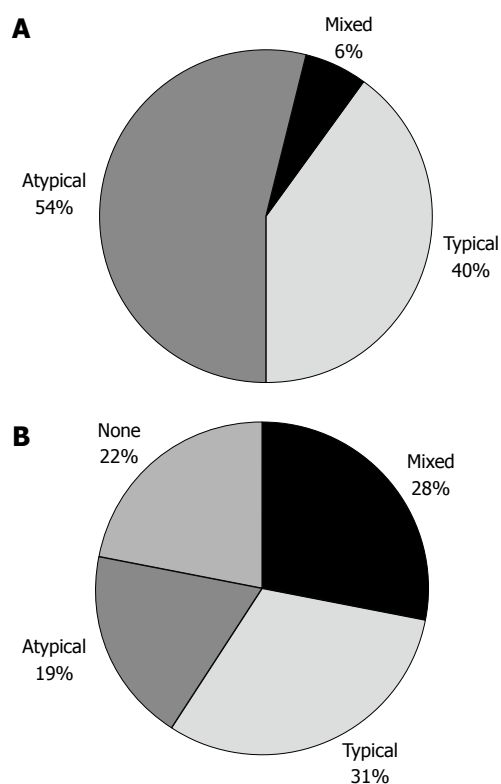


Figure 1 Distribution of gastro-esophageal reflux disease (GERD) symptoms at baseline (A) and at follow-up (B).

Table 2 Variables associated with development of esophagitis in NERD patients: results of the univariate analysis

Risk factor	Odds ratio (95% CI)	P value
Smoking	3.1 (1.6-6.04)	0.0006
Regurgitation > 1/wk	3.5 (1.9-6.6)	0.0001
Severe regurgitation	2.6 (1.35-4.8)	0.005
Nocturnal regurgitation	2.4 (1.2-4.7)	0.01
Supine reflux time > 1.2	5.3 (0.8-38.9)	0.03
Continuing PPI therapy	0.3 (0.1-0.6)	0.001

Note that "continuing PPI therapy" has a negative odds ratio, and can therefore be considered as a protective factor against development of esophagitis. NERD: Nonerosive gastro-esophageal reflux disease; PPI: Proton pump inhibitor.

such as heartburn and regurgitation were still present in 80 patients (31%). The distribution of symptoms at follow-up is presented in Figure 1B.

Frequency and severity of heartburn and regurgitation at follow-up are presented in Figures 2 and 3, respectively. Most patients had received a maintenance treatment during the follow-up period and 181/260 (69.6%) of them were still on therapy; of these, the majority (55%) had been treated with PPIs, 35% had been treated with H₂ receptor agonists and the remaining with non-antiseecretory agents. Among patients treated with PPIs during the last year (100 subjects), 65% were taking a full dose, i.e. the dose usually used for acute therapy, and the remaining 35% were taking a half dose. In either case, only 44% were using a continuous therapy,

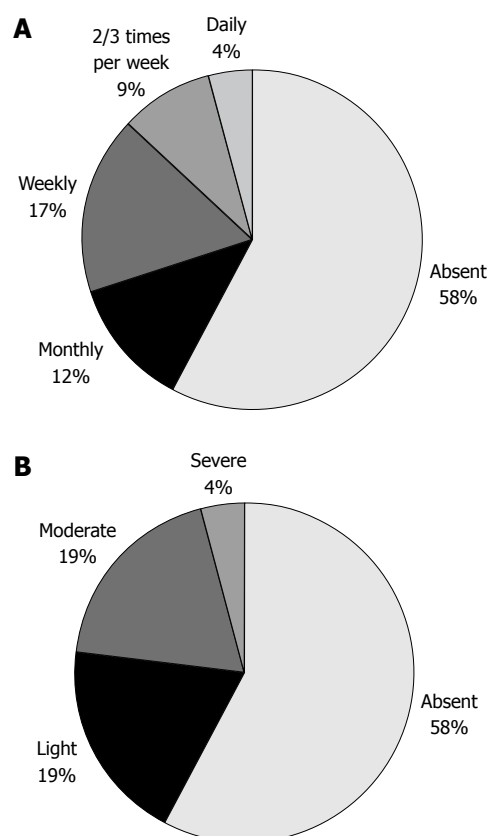


Figure 2 Frequency (A) and severity (B) of heartburn at follow-up. Light: Not interfering with day or night activities; Moderate: Interfering with day or night activities, but bearable; Severe: Unbearable.

whereas the remaining 48% were using an intermittent one and only 8% used a true on-demand therapy. One hundred and ninety-three out of 260 included patients underwent repeat endoscopy during the follow-up period. An average number of 1.5 symptomatic relapses per patient/year of follow-up were observed with an average of 1.6 ± 0.3 (SD) repeat endoscopies performed during the follow-up. Reason for repeat endoscopy was almost always symptom relapse. Despite active therapy, a progression to erosive gastro-esophageal reflux disease (ERD) was found in 58 patients (30.0%) overall; 72% of these cases were Los Angeles grade A or B.

In the univariate analysis, factors found to be significantly associated with the development of esophagitis were smoking, the presence of severe or nocturnal regurgitation and supine reflux time > 1.2% (but not upright or total reflux time), as well as the absence of continuous use of PPI therapy (Table 2). Interestingly, neither the age of the patient nor the BMI value (and in particular a BMI > 30) were found to influence the development of esophagitis. Table 3 shows the pH-metry parameters and the BMI values at presentation of those patients showing a progression vs. those not showing development of esophagitis.

In multivariate logistic regression analysis, only two factors were found to be independent predictors of development of esophagitis: smoking habit and the use of a continuous PPI therapy (Table 4).

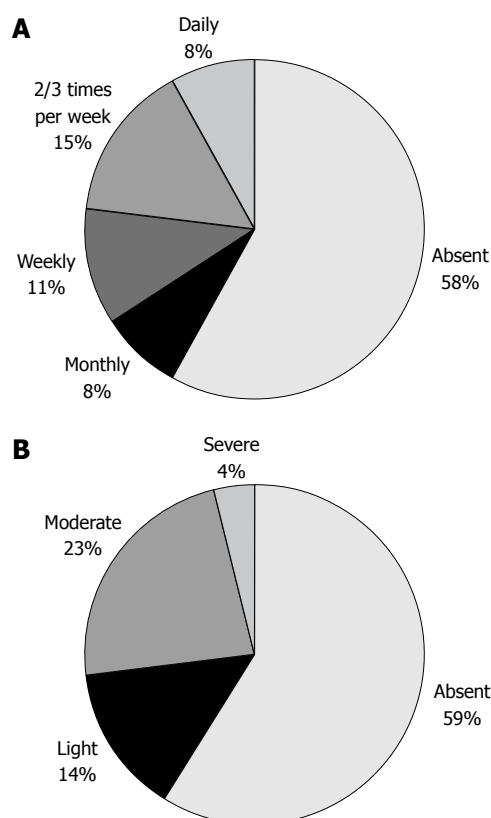


Figure 3 Frequency (A) and severity (B) of regurgitation at follow-up. For explanation of definitions, see legend of Figure 2.

DISCUSSION

This retrospective study clearly shows that during a relatively long follow-up conducted in a sample of patients with NERD and pathological acid reflux, esophagitis develops in a clinically relevant proportion of patients (roughly one quarter); even in those undergoing a maintenance treatment. Smoking and the lack of a continuous PPI therapy are the only factors independently associated with the development of esophagitis, which, in our study, was predominantly grade A or B according to Los Angeles classification.

Our retrospective study is the largest ever conducted in a population of NERD patients further categorized on the basis of a pathological pH-monitoring.

We preferred to include only patients in whom an abnormal acid exposure could be demonstrated by 24-h esophageal pH monitoring for two main reasons: (1) to replicate the inclusion criteria we used in a previously published paper^[11] and (2) to deal with a more homogeneous GERD patient population. In fact, as already demonstrated by others, a significant proportion of presumed NERD patients defined on the basis of symptoms alone do not have GERD^[12]. It has been shown that, on the whole, pH testing is abnormal in about 45% of NERD patients, compared with 75% of patients with erosive esophagitis and 93% of patients with Barrett's esophagus^[13]. Thus, our population sample is not representative of the NERD population at large, but reflects a more homogenous subgroup of NERD patients, which can be defined as having a "true" acid-

Table 3 Baseline reflux parameters of pH-metry and BMI in patients showing a progression at follow-up *vs* those not showing development of esophagitis (mean $\pm$ SD)

	% time of esophageal pH < 4			BMI values (kg/m ²)
	Total	Upright	Supine	
Patients developing esophagitis at follow-up	5.1 $\pm$ 1.6	3.2 $\pm$ 2.6	6.2 $\pm$ 2.4	25.2 $\pm$ 3.6
Patients not developing esophagitis at follow-up	7.2 $\pm$ 5.6	6.2 $\pm$ 7.2	6.9 $\pm$ 8.5	25.3 $\pm$ 0.5
P value	> 0.5	> 0.5	> 0.5	> 0.5

Table 4 Variables associated with development of esophagitis in NERD patients: results of the multivariate analysis (complete model)

Risk factor	Odds ratio (95% CI)	P value
Smoking	2.9 (1.4-6.2)	0.0055
Ongoing PPI therapy	0.2 (0.09-0.7)	0.04

related NERD. In fact, whereas the majority of NERD patients with abnormal pH test have > 75% of heartburn occurring during an acid reflux event, this happens in only 10% of NERD patients with a negative pH test^[13]. The particular selection of our patients may be viewed as a limitation as far as the translational capability of the results. On the other hand, the patients have been more precisely defined than solely on the basis of presence of symptoms and negativity of endoscopic features. The natural history of patients such as ours is at present incompletely known; in a previous retrospective study of a small cohort of pH test-positive NERD patients we demonstrated a progression to erosive esophagitis in 5 out of 33 patients treated over a 6 mo period with antacids and/or prokinetics^[11]. In a subsequent study^[10], we extended the observation time of the original patient group up to a median duration of 10 years. We found that almost all the patients we were able to trace (28/29) became affected by GERD symptoms when antisecretory drugs were discontinued, and therefore the majority (75%) were on such a therapy due to GERD symptoms. In addition, esophagitis was found in the vast majority of subjects in whom repeat endoscopy was performed (18/28). Thus, a considerable proportion of the original patient cohort indeed showed a progression from nonerosive to erosive disease within a long enough (> 5 years) time interval.

Other studies, such as that undertaken by Schindlbeck *et al*^[14] or by McDougall *et al*^[15], support the concept that a progression to esophagitis occurs at least in a proportion of NERD patients with a positive pH test at presentation. These results are in keeping with those recently published by Fullard *et al*^[16], who performed a systematic review of studies conducted on GERD patients. In their review, they found that the annual progression rate for NERD patients (not further characterized by pH monitoring) ranged between 0% and 30%.

The multivariate analysis in our study found only two factors were independent determinants of a negative outcome, i.e. the development of esophagitis; namely

smoking and the lack of a continuous PPI therapy.

These results seem to indicate that the information obtained with esophageal pH-monitoring in NERD patients is not useful to predict the potential progression of such patients toward erosive esophagitis. This has already been observed in the literature in the past; for example, in our previous study^[11] conducted on a small and different patient sample, we found that patients who developed endoscopic esophagitis did not have a more severe baseline pattern of GER pH-metrically measured compared with those who did not develop esophageal mucosal damage. Similarly, Cadiot *et al*^[17], in a multivariate analysis of factors predicting the development of esophagitis in patients with GERD symptoms, found that three independent factors contributed significantly to differentiating between patients with and without esophagitis; namely basal lower esophageal sphincter pressure, peak acid output and the number of reflux episodes during a 24 h period lasting more than 5 min, but not the total esophageal acid exposure time. Also a stepwise regression analysis, conducted on 66 GERD patients and 16 asymptomatic controls, found that hiatal hernia size and basal lower esophageal sphincter pressure, but not esophageal acid exposure or number of reflux episodes during a 24 h period lasting more than 5 min, significantly predicted presence and severity of esophagitis^[18]. Moreover, Labenz *et al*^[19] in a very large prospective study involving more than 6000 GERD patients (The ProGERD Study Initiative) found that no single factor or combination of factors was capable of predicting the presence of esophageal mucosal damage at multivariate analysis. In a case-control study of nearly 5000 patients with and without endoscopic reflux esophagitis investigated with a multivariate regression logistic analysis, Avidan *et al*^[20] found that the only statistically significant factor associated with development of esophagitis was the presence of hiatal hernia. In both studies, however, no pH esophageal recording was performed. Moreover, all these studies were conducted on a mixed GERD population, e.g. patients with and without erosive esophagitis, and aimed at finding factors capable to discriminate these two categories.

Our study followed a different approach; we followed up a cohort of NERD patients and tried to locate factors potentially related to evolution into erosive esophagitis. The only similar study we were able to find in the literature is a study conducted in Japan, in which 47 patients with symptomatic GERD but without esophagitis (NERD) and 450 control subjects were endoscopically investigated at yearly intervals for 5 years, apparently without receiving any antisecretory therapy during this period^[21]. The researchers found that among the group of NERD patients, esophagitis developed in 31.9% as compared with 11.3% in the control group ($P < 0.01$), and that the risk of developing esophagitis was significantly related to absence of *Helicobacter pylori* infection ($P < 0.01$), absence of gastric mucosal atrophy ($P < 0.01$), elevated triglycerides during the 5-year follow-up ($P < 0.05$), and an elevated BMI ($P < 0.05$), with a Cox proportional

hazard model^[21]. This study is however hardly transferable to clinical practice due to exclusion of adequate therapy during the 5-year of follow-up, which in our opinion is an insurmountable ethical drawback of the investigation.

The apparent lack of a relationship between extent of esophageal acid exposure time (and other pathophysiologic parameters of reflux and of esophageal motility) and development of esophagitis has been taken into consideration recently^[22], and it has been suggested that the majority of investigations conducted so far have overlooked the resistance of the esophageal mucosa. Although the effect of smoking on GERD symptoms and lesions is not clear-cut, it probably acts on different pathogenetic sites, including mucosal resistance. Smoking may in fact promote GER by several mechanisms including; attenuation of the tone at the lower esophageal sphincter^[23,24], a decrease in salivary flow and bicarbonate secretion with a resultant prolongation of acid clearance^[25], and a delay in gastric emptying^[26,27]. Some additional effects of smoking and nicotine that may be relevant to GERD are; an increase in gastric acid and pepsin secretion, augmentation of duodeno-gastric bile reflux and attenuation of the protective mechanisms of the gastric mucosa, such as synthesis of prostaglandins, mucus and epidermal growth factor^[28]. Finally, in a recent study conducted on a NERD population which included a pathological pH investigation, smoking was found to be an independent predictor of GERD symptoms at multiple linear regression analysis^[29].

In conclusion, our study shows that progression to ERD occurs in a considerable proportion of NERD patients (close to 5% per year) despite standard therapy, whereas symptomatic relapse occurs more frequently than once per year. Factors consistently and independently influencing this progression are smoking and the lack of a continuous PPI therapy during the follow-up period.

COMMENTS

Background

The natural history of nonerosive gastro-esophageal reflux disease (NERD) is not entirely known. In particular, data are lacking regarding the subgroup of NERD patients with abnormal esophageal pH monitoring. The authors conducted a study to assess the fate of these patients who were followed for a mean of 5 years after the index evaluation.

Research frontiers

To evaluate by means of a multivariate analysis potential factors leading to a progression of the disease, e.g. the development of mucosal lesions.

Innovations and breakthroughs

The present study showed that progression to erosive gastro-esophageal reflux disease occurred in about 5% of their NERD patients per year, despite therapy. Only two factors consistently and independently influenced progression: smoking and absence of proton pump inhibitor (PPI) therapy.

Applications

Due to the clinically relevant number of NERD patients evolving to esophagitis, it is conceivable that the present guidelines for endoscopy in gastro-esophageal reflux disease (GERD) should be changed. In particular, the authors propose the repetition of upper gastrointestinal (GI) endoscopy at least once every 5 years.

Peer review

The authors have conducted a retrospective study which provides useful information regarding the natural history of nonerosive reflux disease patients and the potential factors leading to esophageal mucosal lesions over 5 years of follow-up.

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BRIEF ARTICLE

Impact of age-related comorbidity on results of colorectal cancer surgery

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Abstract

AIM: To analyze the correlation between preexisting comorbidity and other clinicopathological features, short-term surgical outcome and long-term survival in elderly patients with colorectal cancer (CRC).

METHODS: According to age, 403 patients operated on for CRC in our department were divided into group A (< 70 years old) and group B ($\geq$ 70 years old) and analyzed statistically.

RESULTS: Rectal localization prevailed in group A (31.6% vs 19.7%, $P = 0.027$), whereas the percentage of R0 resections was 77% in the two groups. Comorbidity rate was 46.2% and 69.1% for group A and B, respectively ($P < 0.001$), with a huge difference as regards cardiovascular diseases. Overall, postoperative morbidity was 16.9% and 20.8% in group A and B, respectively ($P = 0.367$), whereas mortality was limited to group B (4.5%, $P = 0.001$). In both groups, patients who suffered from postoperative complications had a higher overall comorbidity rate, with preexisting cardiovascular diseases prevailing in group B ($P = 0.003$). Overall 5-year survival rate was significantly better

for group A (75.2% vs 55%, $P = 0.006$), whereas no significant difference was observed considering disease-specific survival (76.3% vs 76.9%, $P = 0.674$).

CONCLUSION: In spite of an increase in postoperative mortality and a lower overall long-term survival for patients aged $\geq$ 70 years old, it should be considered that, even in the elderly group, a significant number of patients is alive 5 years after CRC resection.

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Key words: Colorectal cancer; Elderly; Post-operative complications; Co-morbidity; Aged

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INTRODUCTION

Colorectal cancer (CRC) generally is considered a disease of the elderly^[1] because of its rising incidence with age^[2-6]. It represents an important cause of morbidity and mortality in the elderly population^[1], which mainly undergo emergency surgery because of bowel obstruction or perforation^[1,2,7-9]. Several authors have reported that elderly patients with CRC can be treated by standard surgical resection and surgery should not be denied on account of chronological age^[1,3,8,10,11]. Comorbidity, previously different tumors, and poor performance status represent the early and late negative prognostic factors in elderly patients^[1,2,7,10,12-15]. The purpose of this observational study was to evaluate our experience with CRC, to assess surgical and prognostic features of patients aged $\geq$ 70 years compared with younger patients, with special reference to related comorbidity.

MATERIALS AND METHODS

Population under study

Between January 1999 and March 2006, 420 patients with CRC were observed in our department. Among these, seven patients were not operated on because of their poor general status or advanced stage of disease, and 10 patients were lost at follow-up. Thus, 403 patients (229 male and 174 female) with a mean ($\pm$ SD) age of 67.2 ± 11.5 years (range 23–98 years) were analyzed retrospectively. According to the age at operation, two groups were considered: group A (< 70 years old; 225 patients) and group B (≥ 70 years old; 178 patients).

Preoperative assessment and staging

Upon admission, all patients were studied with colonoscopy, abdominal ultrasonography and computed tomography (CT) to gain a correct preoperative staging. All comorbidity was noticed and patients underwent clinical and instrumental examinations. Cardiac diseases were assessed as electrocardiographic or echocardiographic abnormalities, or pathology for which the patient was under specific treatment; vascular pathology as cases of hypertension treated with specific drugs; cerebrovascular pathology; pulmonary diseases as abnormal spirometry, or pathology for which the patient was taking medication. Surgical risk was evaluated preoperatively with ASA score by a senior anesthesiologist. Antibiotic and thromboembolic prophylaxis was performed in all patients. The presence of residual tumor (R category) and tumor stage (TNM) were defined on the basis of the criteria established by the Union International Contre Le Cancer (UICC).

Surgical approach and techniques

The main goal of surgery was the complete removal of the tumour (R0 resection), although palliative resection was performed to treat tumor-related complications. Right and left hemicolectomy, low or very low anterior resection, or transverse colon resection were carried out as standard techniques for CRC, with vessel ligation and respecting oncological criteria. In the event of multiple and synchronous localization, the extent of resection was chosen case by case. Hartmann resection was performed rarely. Definitive loop colostomy or ileostomy was reserved for advanced tumors or patients with very bad performance status in the event of severe bowel obstruction. Temporary covering ileostomy at primary surgery was planned mainly for very low anterior resection for rectal cancer.

Follow-up and statistical analysis

All clinical and surgical data were collected and stored in a PC database. The data concerning follow-up were collected during outpatient clinical examination and by contacting the family physician. The collected data included demographic and clinical characteristics, co-morbidity (cardiovascular, pulmonary and digestive diseases, diabetes mellitus and other tumors), site of neoplasm (colon or rectum), type of surgery (elective or emergency), presence of residual tumour (R0 or R1–2 resection), histological features, tumour stage (TNM) and Dukes' classification,

as well as postoperative morbidity and mortality. A particular effort was made to analyze comorbidity, postoperative complications and survival rates, to identify specific features of the two groups under study. Cardiovascular diseases included heart disease such as myocardial ischemia or valvular disease, both with hypertension and cerebrovascular disorders; and aortic as well as peripheral arterial disorders were also included. Digestive comorbidity included major gastrointestinal, hepatic or biliary-pancreatic diseases such as inflammatory bowel disease, pancreatitis, hepatic cirrhosis and portal hypertension. We also focused on patients who were affected by tumors other than CRC, including them in the "other tumor" comorbidity group. In assessing postoperative complications and mortality, events that occurred during hospitalization or within 30 d after operation were included in the analysis. Diseases such as pulmonary embolism, myocardial ischemia or acute renal failure, and surgical complications, such as anastomotic leakage or abdominal infection, were considered separately. Statistical differences in clinicopathological characteristics and comorbidity between the groups were assessed by the χ^2 test for categorical variables and Student's *t* test for continuous variables. Survival curves were calculated according to the Kaplan-Meier model. Survival curves were calculated for the two groups with regard to overall and disease-specific survival; operative mortality was calculated in the analysis. The log-rank test was used to assess the difference between the two groups. $P < 0.05$ was considered significant.

RESULTS

Clinicopathological findings

Clinicopathological features and presence of comorbidity in the two groups are reported in Table 1. Among the 403 patients, we observed 106 tumors that arose from the rectum, 109 from the right colon, 20 from the transverse colon, and 160 from the descending or sigmoid colon. Multiple tumors were observed in eight cases. Rectal tumors prevailed in group A (31.6% *vs* 19.7%, $P = 0.027$); otherwise, topographic distribution of colon cancer was rather homogeneous between the two groups. Elderly patients underwent emergency operation more frequently compared to younger ones, although the difference was not statistically significant (14% *vs* 9.3%, $P = 0.094$). A potentially curative (R0) resection was achieved with a rate of about 77% in both groups ($P = 0.512$). With respect to the Dukes' and TNM classifications, no significant differences were noted between groups A and B. As regards comorbidity, our findings suggest a significant difference between group A and B with an overall rate of 46.2% and 69.1%, respectively ($P < 0.001$). By analyzing different comorbidity in each group, we found that patients aged ≥ 70 years had a 51.1% rate of cardiovascular diseases compared to 30.7% in the younger ones ($P < 0.001$). Weak differences were noticed between group A and B with respect to pulmonary diseases ($P = 0.048$) and other tumors ($P = 0.050$). Other comorbidity did not show any significant features in either of the populations studied.

Table 1 Clinical and pathological data for group A (< 70 years) and B (≥ 70 years) *n* (%)

	Group A (<i>n</i> = 225)	Group B (<i>n</i> = 178)	<i>P</i>
Sex			
Males	126 (56)	103 (57.9)	0.392
Females	99 (44)	75 (42.1)	
Localization ¹			
Colon	152 (67.6)	141 (79.2)	0.027
Rectum	71 (31.6)	35 (19.7)	
Type of surgery			
Elective	204 (90.7)	153 (86)	0.094
Emergency	21 (9.3)	25 (14)	
Residual tumor			
R0	174 (77.3)	137 (77)	0.512
R+	51 (22.7)	41 (23)	
Comorbidity			
Overall	104 (46.2)	123 (69.1)	0.0001
Cardiovascular diseases			
Absent	156 (69.3)	87 (48.9)	0.0001
Present	69 (30.7)	91 (51.1)	
Pulmonary diseases			
Absent	217 (96.4)	164 (92.1)	0.048
Present	8 (3.6)	14 (7.9)	
Diabetes mellitus			
Absent	211 (93.8)	160 (89.9)	0.106
Present	14 (6.2)	18 (10.1)	
Gastrointestinal diseases			
Absent	198 (88)	158 (88.8)	0.470
Present	27 (12)	20 (11.2)	
Other tumors			
Absent	216 (96)	163 (91.6)	0.050
Present	9 (4)	15 (8.4)	
Dukes' stage			
A	14 (6.2)	8 (4.5)	0.623
B	96 (42.6)	89 (50)	
C	64 (28.4)	41 (23)	
D	51 (22.7)	40 (22.5)	
T stage			
T1	18 (8)	10 (5.6)	0.485
T2	36 (16)	35 (19.7)	
T3	148 (65.8)	110 (61.8)	
T4	23 (10.2)	23 (12.9)	
N stage			
N0	122 (54.2)	110 (61.8)	0.270
N1	64 (28.4)	45 (25.3)	
N2	39 (17.3)	23 (12.9)	
Metastases			
No	174 (77.3)	138 (77.5)	0.530
Yes	51 (22.7)	40 (22.5)	

¹Synchronous localizations of colon and rectum are represented in two cases for each group (0.9% vs 1.1%).

Short-term postoperative results

The distribution of surgical procedures is reported in Table 2. Hartmann resection and palliative stoma were performed rarely, and mostly they were carried out in elderly patients. Concerning the postoperative period, overall morbidity was 16.9% and 20.8% in group A and B, respectively ($P = 0.367$). No significant difference was observed as regards surgical complications, whereas medical adverse events were twofold greater in group B compared to group A (9% vs 4.8%, $P = 0.122$). Among cardiovascular complications, acute heart failure or pulmonary edema was the most common postoperative morbidity. Among surgical complications, the percentage

Table 2 Surgical procedures and postoperative results *n* (%)

	Group A (<i>n</i> = 225)	Group B (<i>n</i> = 178)	<i>P</i>
Postoperative mortality	-	8 (4.5)	0.001
Postoperative complications	38 (16.9)	37 (20.8)	0.367
Surgical complications	27 (12)	21 (11.8)	1
Medical complications	11 (4.9)	16 (9)	0.112
Operation			
Right hemicolectomy	53 (23.6)	61 (34.3)	
Left hemicolectomy	73 (32.4)	50 (28.1)	
Transverse colon resection	5 (2.2)	9 (5.1)	
Abdominoperineal resection	9 (4)	8 (4.5)	
Lower anterior resection	78 (34.7)	43 (24.2)	
Hartmann resection	1 (0.4)	4 (2.2)	
Stoma	2 (0.9)	2 (1.1)	
Miscellaneous	4 (1.8)	1 (0.6)	
Anastomotic leakage rate	10 (4.4)	4 (2.3)	0.282

with anastomosis leakage was 3.5%, with a higher number of cases in group A (4.4% vs 2.3%, $P = 0.282$). Overall postoperative mortality was 2% and it was limited to patients aged ≥ 70 years (4.5%, $P = 0.001$) (Table 2).

Preexisting comorbidity and short-term results

Table 3 shows the correlation between clinicopathological features, preexisting comorbidity and the occurrence of postoperative complications. The overall comorbidity rate was significantly higher in patients who developed postoperative complications in groups A and B. In group A, male patients experienced complications more frequently than females ($P = 0.049$). In group B, preexisting cardiovascular diseases were significantly associated with postoperative complications ($P = 0.003$). Other features did not influence significantly the postoperative course.

Survival analysis

The 5-year survival rate for all 403 patients was 62.9%, whereas it was 76.4% for the R0-resected patients. Considering all causes of death, the overall 5-year survival rate was significantly better for group A (75.2% vs 55%, $P = 0.006$) (Figure 1). Conversely, considering disease-specific survival (postoperative and tumor-related deaths only), no difference was observed between groups A and B (76.3% vs 76.9%, $P = 0.674$) (Figure 2).

DISCUSSION

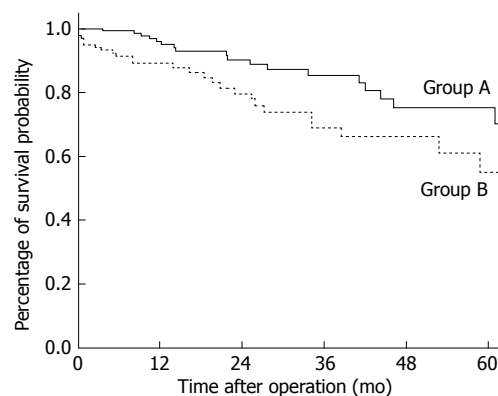
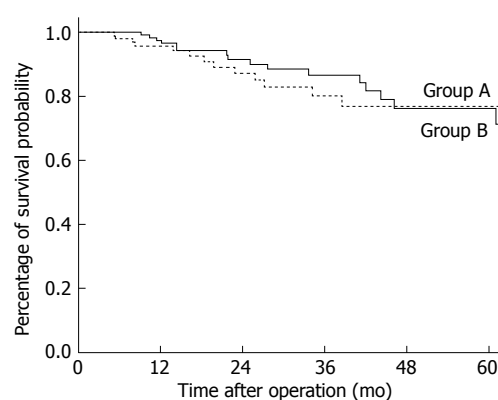
CRC in the elderly

As for other tumors, the development of CRC is associated with increasing age. The reason for cancer development in the elderly can be explained by a longer duration of exposure to carcinogens, a lower ability to repair damaged DNA, and oncogene amplification or tumor suppressor gene malfunction. Furthermore, the progressive loss of immune surveillance can be considered to occur with aging^[8,16]. Despite progress in surgical and perioperative care, a lot of physicians evaluate elderly patients by their chronological rather than biological age^[17]. This attitude may explain why treatment is frequently conditioned in elderly people with malignancies. As a consequence, their therapeutic

Table 3 Correlation between clinicopathological features and preexisting comorbidity with postoperative complications in group A (< 70 years) and B ($\geq$ 70 years) *n* (%)

	Postoperative morbidity			
	Group A (<i>n</i> = 38)	<i>P</i>	Group B (<i>n</i> = 37)	<i>P</i>
Sex				
Male	27 (71.1)	0.049	23 (62.2)	0.58
Female	11 (28.9)		14 (37.8)	
Localization				
Colon	23 (60.5)	0.344	26 (70.3)	0.171
Rectum	15 (39.5)		11 (29.7)	
Type of surgery				
Elective	32 (84.2)	0.136	30 (81.1)	0.424
Emergency	6 (15.8)		7 (18.9)	
Residual tumor				
R0	27 (71.1)	0.297	29 (78.4)	0.505
R+	11 (28.9)		8 (21.6)	
Comorbidity				
Overall				
Yes	25 (65.8)	0.012	32 (86.5)	0.01
No	13 (34.2)		5 (13.5)	
Cardiovascular diseases				
Absent	22 (57.9)	0.122	10 (27)	0.003
Present	16 (42.1)		27 (73)	
Pulmonary diseases				
Absent	36 (94.7)	0.625	32 (86.5)	0.172
Present	2 (5.3)		5 (13.5)	
Diabetes mellitus				
Absent	34 (89.5)	0.264	34 (91.9)	0.769
Present	4 (10.5)		3 (8.1)	
Gastrointestinal diseases				
Absent	30 (78.9)	0.095	32 (86.5)	0.571
Present	8 (21.1)		5 (13.5)	
Other tumors				
Absent	36 (94.7)	0.65	32 (86.5)	0.314
Present	2 (5.3)		5 (13.5)	
T stage				
T1-T2	5 (13.2)	0.098	12 (32.4)	0.29
T3-T4	33 (86.8)		25 (67.6)	
N stage				
N0	15 (39.5)	0.051	23 (62.2)	0.559
N+	23 (60.5)		14 (37.8)	
Dukes' stage				
A	1 (2.6)	0.385	3 (8.1)	0.669
B	13 (34.2)		17 (45.9)	
C	13 (34.2)		9 (24.3)	
D	11 (28.9)		8 (21.6)	
Metastases				
No	27 (71.1)	0.297	29 (78.4)	0.542
Yes	11 (28.9)		8 (21.6)	

management remains controversial. As a result of their age, elderly people usually suffer from other chronic illnesses in addition to colorectal malignancy. Nonetheless, surgical resection remains the treatment of choice, probably because surgery still represents the mainstay of therapy for CRC, and the most used option to treat bowel obstruction. In a recent study by Lemmens *et al*^[18], it has been demonstrated that age > 70 years, a tumor located in the rectum, emergency surgery, and the presence of concomitant chronic obstructive pulmonary disease or deep vein thrombosis increase the risk of developing a surgical complication. On the contrary, CRC patients without comorbidity developed surgical complications in < 30% of the cases.

**Figure 1** Kaplan-Meier estimates of survival probability for R0 population in group A (< 70 yr; 174 pts) and B ($\geq$ 70 yr; 137 pts). Postoperative, tumor-related and -unrelated deaths were included (Log-rank test, *P* = 0.006).**Figure 2** Kaplan-Meier estimates of disease-specific survival probability for R0 population in group A (< 70 yr; 174 pts) and B ($\geq$ 70 yr; 137 pts). Postoperative and tumor-related deaths were included (Log-rank test, *P* = 0.674).

Postoperative morbidity

With reference to our results, we had a comparable postoperative morbidity rate^[19], with no statistically significant difference between the two groups. Considering medical complications, elderly patients had a twofold greater, even though not significant, rate of adverse events such as pulmonary embolism and myocardial ischemia. This may be due to a higher prevalence of cardiovascular diseases at the time of cancer diagnosis. Our previous study supports these data that show that cardiac complications are the most frequent in octogenarians affected by gastrointestinal carcinoma^[20,21]. Based upon our findings, cardiovascular comorbidity has a significant influence on the development of postoperative complications in elderly as well as younger patients. As regards postoperative complications, we observed a low rate of complications with no difference between the two groups. Our data seem to confirm the possibility of achieving a very low percentage of adverse events in aged patients, with curative resection and overall anastomotic leakage rates equally successful in both groups.

Comorbidity and postoperative complications

Current literature supports the notion that a higher mortality rate in older patients can be attributed mainly to preexisting comorbid conditions^[10,22], such as congestive

heart failure, diabetes mellitus and chronic obstructive pulmonary disease^[23]. It also emphasizes the role of interactions between disease conditions and CRC, and suggests that multiple comorbidity has a substantial effect on long-term survival. The number of comorbid conditions increases with age and the most frequent diseases are hypertension, other cardiovascular diseases and previous malignancy^[1,12]. In agreement with other experiences, male patients have a higher prevalence of postoperative complications compared to females^[24], and an explanation may be found in the lower number of women suffering from cardiovascular diseases, especially at younger ages. Our data confirmed that elderly patients, in spite of an equal percentage of postoperative complications, have a higher mortality rate compared to younger patients^[7,10,25,26]. The relationship between preoperative comorbid conditions and postoperative mortality has been demonstrated by previous studies^[10,23,27-30]. Moreover, we assessed that in most of the eight patients who died, there was more than one comorbid condition. These results suggest that postoperative complications are tolerated poorly by elderly patients, particularly those suffering from comorbidity.

Overall and tumor-related survival

In the elderly population with CRC, the relationship between age and long-term outcome after surgery remains not yet completely defined, and the results on survival are still a matter of debate, with alternative conclusions^[3,7]. Regarding overall survival, it has been demonstrated that it decreases with aging^[1]. Nonetheless, disease stage influences specific tumor survival independently of age. It has been reported that the decrease in survival is more evident in very old patients, particularly those aged > 85 years. In this regard, our findings clarify that elderly people have a significantly worse overall survival rate compared to younger ones, but, after censoring cancer-unrelated deaths, survival rates become comparable. Thus, strictly considering tumor-related mortality, no difference was observed between the two groups. Other authors have supported our results^[2] and have emphasized the relationship between early stage of the disease and a similar prognosis in elderly compared to younger patients. In spite of an increase in postoperative mortality and a shorter overall long-term survival for patients aged $\geq$ 70 years, it should be considered that, even among elderly patients, a significant number of patients is alive 5 years after CRC resection.

In conclusion, in agreement with previous studies, our findings suggest that a relevant positive outcome after a potentially curative resection should encourage surgical treatment of elderly patients with CRC. Comorbidity represents a risk factor for developing postoperative complications in younger and older patients. A higher risk of postoperative mortality seems to be a prerogative of elderly patients affected by other comorbid conditions, especially cardiovascular diseases. As a consequence, surgeons should be more cautious when confronted with elderly patients suffering from severe comorbidity. Even if treatment decisions in elderly patients with CRC should be

made on the basis of careful evaluation of cardiovascular and pulmonary parameters, surgical tumor resection clearly is encouraged by high rates of potentially curative resection and satisfactory long-term survival results.

COMMENTS

Background

Colorectal cancer (CRC) generally is considered to be a disease of the elderly. Several studies have reported that elderly patients with CRC can be treated by standard surgical resection, although some debate still exists on this topic.

Research frontiers

The correlation between preexisting comorbidity, clinicopathological features, and short- and long-term outcomes was studied in elderly patients undergoing surgery for CRC.

Innovations and breakthroughs

A particular effort has been made to analyze preexisting comorbidity, postoperative complications and long-term results, to identify features specific for elderly patients compared to younger ones.

Applications

In spite of an acceptable increase in postoperative mortality and a shorter overall long-term survival, surgical resection provides encouraging results for patients aged $\geq$ 70 years.

Peer review

The authors investigated the relationship between preexisting diseases and other clinicopathological features, perioperative surgical outcome, and survival in elderly patients with CRC treated by surgery. The conclusions are definite, and the contents are well-organized and well-written. The results give us some new ideas about the elderly population undergoing surgery for CRC.

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BRIEF ARTICLE

Effect of ONO-4057 and tacrolimus on ischemia-reperfusion injury of the liver

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Abstract

AIM: To investigate the effects of a novel Leukotriene B₄ receptor antagonist and/or tacrolimus on ischemia-reperfusion in a rat liver model.

METHODS: Male Lewis rats were pretreated with ONO-4057 (100 mg/kg) and/or tacrolimus (1 mg/kg) orally, and divided into four experimental groups; group 1 (control), group 2 (ONO-4057), group 3 (tacrolimus), group 4 (ONO-4057 + tacrolimus).

RESULTS: There was a tendency for long survival in the groups treated with tacrolimus alone and ONO-4057 plus tacrolimus. Post-reperfusion serum aspartate aminotransferase levels decreased more significantly in ONO-4057 plus tacrolimus group ($P < 0.01$), than in the tacrolimus alone group ($P < 0.05$), compared to controls.

CONCLUSION: This study demonstrated that pretreat-

ment with ONO-4057 in combination with tacrolimus produced additive effects in a rat model of liver ischemia-reperfusion injury.

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Key words: Ischemia-reperfusion injury; Leukotriene B₄; Liver; Tacrolimus

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Takeichi T, Uemoto S, Minamiguchi S, Takeyoshi I, Inomata Y, Tanaka K, Kobayashi E. Effect of ONO-4057 and tacrolimus on ischemia-reperfusion injury of the liver. *World J Gastroenterol* 2009; 15(45): 5712-5715 Available from: URL: <http://www.wjg-net.com/1007-9327/15/5712.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5712>

INTRODUCTION

Neutrophils are mainly responsible for the physiopathological changes that occur after ischemia-reperfusion injury^[1-3]. These cells are markedly activated when the ischemically damaged liver is transplanted. Experimental efforts have focused on understanding the etiology of ischemic injury and protecting the liver by pharmacological intervention^[4-7].

Pretreatment with certain immunosuppressive drugs has been shown to protect against neutrophil-mediated reperfusion injury^[4,5]. Calcineurin inhibitors are thought to suppress both proinflammatory cytokine production and expression of adhesive molecules of neutrophils^[8]. Recently, ONO-4057 (5-[2-(2-carboxyethyl)-3-{6-(4-methoxyphenyl)-5E-hexenyl}yphenoxy]valeric acid), an orally administered active leukotriene B₄ (LTB₄) receptor antagonist, was developed and was shown to have an important effect on LTB₄-induced neutrophil function^[6,7].

In this study, we investigated the effect of pretreatment with ONO-4057 alone and in combination with tacrolimus on ischemia-reperfusion injury of the liver in rats.

MATERIALS AND METHODS

Animals

Male Lewis rats (Charles River, Hamamatsu, Japan),

5-6 wk of age, were used in this study. The animals were housed in a pathogen-free, temperature- and light-controlled environment with free access to food and water. The rats were anesthetized with ether for induction and maintenance. All experimental protocols were approved by the institutional review board of Kyoto University.

Experimental groups

The animals were divided into four experimental groups: group 1, controls; group 2, ONO-4057; group 3, tacrolimus; and group 4, ONO-4057 and tacrolimus ($n = 9$ in each group). The control groups received distilled water (DW) and NaHCO_3 as a vehicle administered orally, and the other groups were pretreated with ONO-4057 (Ono Pharmaceutical Co., Ltd., Osaka, Japan) and tacrolimus (Fujisawa Pharmaceutical Co., Osaka). ONO-4057 was dissolved to a concentration of 30 mg/mL with NaHCO_3 , and tacrolimus was dissolved to a concentration of 0.3 mg/mL with DW. ONO-4057 was administered orally at a dose of 100 mg/kg and tacrolimus orally at a dose of 1 mg/kg. All agents were administered 1 h before ischemia was induced.

Ischemia-reperfusion model

Whole-liver ischemia model: Normothermic ischemia of the entire liver (with intestinal congestion) was induced as described previously^[8]. Briefly, a midline incision was made after the animals were anesthetized with ether and the portal vein, hepatic artery, and bile duct were clamped for 40, 50 and 60 min with a vascular micro-clip. The clip was then released and the abdominal wall was closed using a continuous running suture. The animals were returned to their cages for survival analysis for the following 2 wk.

Partial-liver ischemia model: To obtain data about more severe normothermic ischemia damage of the liver without intestinal congestion, the left and middle portal vein and hepatic artery were occluded for 60 min according to the procedure of Kawano *et al.*^[9] with a minor modification. This procedure induced 70% warm-ischemic liver damage, but 30% healthy liver remained to protect the animal's life. After reperfusion, the abdominal wall was closed, and the treated rats were observed. One mL of the peripheral blood were obtained at 3 and 6 h after de-clamping, then the liver was perfused with 10 mL cold saline and immediately taken out. The damaged part of the liver was used for histological assessment and measurement of liver-tissue myeloperoxidase (MPO).

Serum measurements

Using the peripheral blood obtained from the experimental animals, serum transaminase concentrations [aspartate aminotransferase (AST); alanine aminotransferase (ALT)] were measured by an ultraviolet method using an automatic analyzer (Hitachi 7170, Tokyo, Japan).

Histological examination

Hepatic samples were fixed in 10% buffered formaldehyde,

Table 1 Survival rates with various pretreatments

Duration of ischemia (min)	Survival rates (%)			
	Controls	ONO-4057	Tacrolimus	ONO-4057 + tacrolimus
40	6/6 (100)	6/6 (100)	NT	NT
50	6/6 (100)	6/6 (100)	NT	NT
60	2/9 (22)	4/9 (44)	6/9 (66)	5/9 (56)

NT: Non-tested.

embedded in paraffin, cut to 3 to 5 μm thick, and stained with hematoxylin and eosin. The specimens were blindly analyzed to evaluate the histological damage according to the modified classification of Suzuki *et al.*^[4].

Liver-tissue MPO activity: MPO tissue level is considered to be an index of neutrophil infiltration in the liver. Liver samples obtained at 6 h after reperfusion were frozen in liquid nitrogen and stored at -70°C until the time of measurement. Tissue samples were then homogenized in 10 mL phosphate-buffered saline and centrifuged at 100 000 g and 4°C for 30 min. The resultant supernatant was assayed for MPO activity using the modified method of Shindler *et al.*^[10]. Briefly, the reaction mixture, containing 10:1 of supernatant and 200:1 of 100 mol/L ABTS buffer with H_2O_2 , was incubated in 96-well plate for 60 min at 25°C . The optical density at 414 nm was determined by a plate reader (SPECTRA maxTM 340; Molecular Devices Co., CA, USA). The MPO activity was calculated using an extinction coefficient of $(3.6 \times 10^4)/(\text{mol per cm})$ for ABTS and normalized to protein levels ($\mu\text{mol}/\text{mg}$ protein). The protein concentration was determined by the method of Lowry *et al.*^[11].

Statistical analysis

The repeated measurement analysis of variance (ANOVA) was performed among the groups. If repeated measurement ANOVA revealed a significant interaction, the statistical significance among the four groups at each time point was determined using post hoc tests. A P value of less than 0.05 was considered statistically significant.

RESULTS

Survival after pretreatment with ONO-4057 and/or tacrolimus on whole-liver ischemia in rats is shown in Table 1. Because 40 to 50 min of whole liver ischemia in adult Lewis rats (about weighting 280-330 g) was sublethal, we initially evaluated the 40 to 50 min ischemic damage in young Lewis rats in this study. However, all rats that had undergone 40 or 50 min of whole-liver ischemia survived indefinitely. When the results of 60 min of ischemia were evaluated, there was no significant difference in survival rate among the groups. However, the survival rate was slightly higher in the groups treated with tacrolimus alone ($P = 0.053$) and ONO-4057 plus tacrolimus. Serum AST values at 3 and 6 h after partial-liver ischemia-reperfusion are shown in Figure 1A. In the group pretreated with tacrolimus alone, the serum

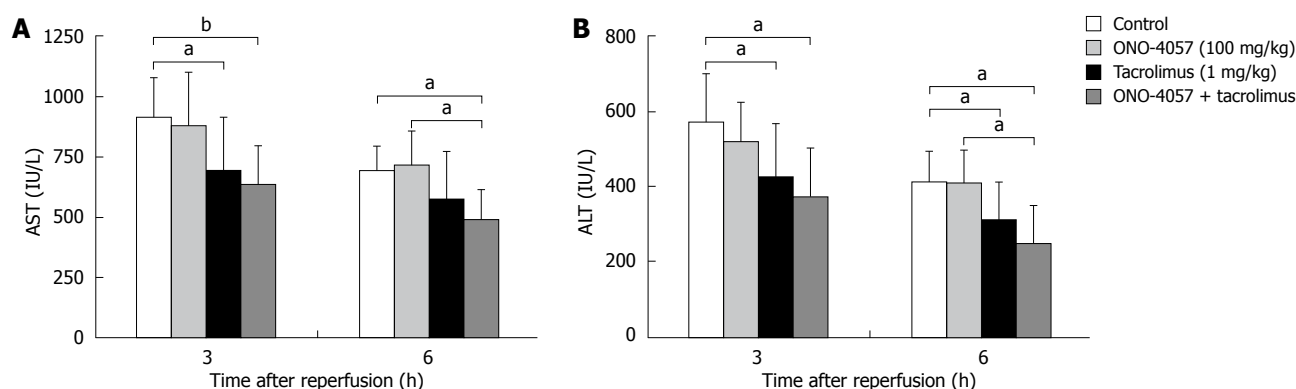


Figure 1 Serum transaminase levels after reperfusion. A: Aspartate aminotransferase (AST) levels after partial-liver ischemia for 60 min; B: Alanine aminotransferase (ALT) levels after partial-liver ischemia for 60 min. ^a $P < 0.05$, ^b $P < 0.01$.

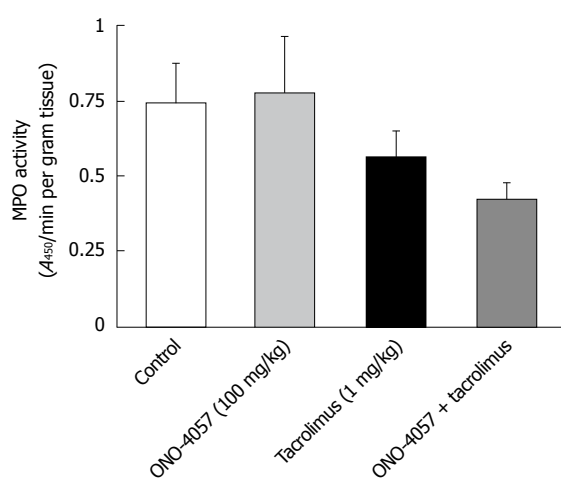


Figure 2 Myeloperoxidase (MPO) activity after reperfusion.

AST value was significantly lower than the control group at 3 and 6 h after reperfusion ($P < 0.05$), respectively. In the group pretreated with ONO-4057 plus tacrolimus, the AST level was more significantly lower compared with the control group ($P < 0.01$). Pretreatment with ONO-4057 and tacrolimus produced additive effects, but not when compared with the group treated with tacrolimus alone. ONO-4057 alone could not decrease the transaminase levels. Serum ALT values demonstrated a similar pattern (Figure 1B).

The liver MPO levels after 6 h of reperfusion are shown in Figure 2. There was no significant difference among the groups in MPO activity. However, the MPO level in the group treated with ONO-4057 plus tacrolimus tended to be lower compared with that in the control group, although it was not statistically significant ($P = 0.059$). At 6 h from the reperfusion after 60 min of partial liver ischemia, the liver damage (congestion, vacuolization, and necrosis) that was observed histologically was not significantly different among the groups.

DISCUSSION

In liver transplantation, ischemia-reperfusion injury is one of the major factors that might influence early graft function and late changes. The importance of

neutrophils in the development of ischemia-reperfusion injury in the liver has been demonstrated^[12,13].

LTB₄, a metabolite formed *via* the 5-lipoxygenase pathway from arachidonic acid, is one of the most potent chemotactic and proinflammatory mediators. LTB₄ causes neutrophil adhesion to vascular endothelial cells and infiltration into vascular endothelial cells. We investigated the effect of the LTB₄ receptor antagonist, ONO-4057, on hepatic ischemia-reperfusion injury to determine whether they affect neutrophil infiltration into the liver and attenuate neutrophil-induced post-ischemic injury. Recent reports have also shown that some immunosuppressive drugs protect or attenuate the neutrophil-mediated ischemia-reperfusion injury in various organs (liver, small intestine, kidney, and heart)^[5,14,15]. As tacrolimus has variable effects^[16], we tested the effect of a combination of ONO-4057 with tacrolimus. The results showed that ischemia-reperfusion injury in the liver was not reduced by pretreatment with ONO-4057 alone. Tacrolimus was necessary for protection from I/R injury. Pretreatment with ONO-4057 had additive effects on the protective function of tacrolimus. Although the I/R damage was significantly prevented with the use of Tac and ONO-4057, the main damage might be related directly to the role of immunosuppressants. The suppression of the elevation of transaminases after I/R in this experiment might be mainly caused by the effect of tacrolimus. The MPO level in the liver specimens and the blood level of the transaminases did not correlate well in this study. MPO levels might be affected sub-significantly by the ONO-4057-mediated modification of neutrophils infiltration, but it did not directly prevent the hepatic damage.

Hepatic ischemia-reperfusion injury is caused by many factors, and this study has limited because it only focused on neutrophils' infiltration. Two main causes have been reported for I/R injury; oxygen free radicals and neutrophil-mediated damage. Recent reports showed a beneficial effect of neutrophil-mediated inhibitors, such as neutrophil elastase inhibitor and tissue factor pathway inhibitor, on hepatic reperfusion injury^[17,18]. In another study, treatment with leukotriene biosynthesis inhibitor (L664,536) had no effect on hepatic injury, although LTB₄ formation was inhibited^[19]. We showed the added effect

of a LTB₄ receptor antagonist and immunosuppressive drug. As ONO-4057 has been also reported to have an immunosuppressive effect in allogenic rat liver grafts^[20], co-administration of ONO-4057 and tacrolimus might be reasonable for liver transplantation. Before any clinical trials, experiments using the transplantation model might be necessary.

In conclusion, our data showed that pretreatment with ONO-4057 in combination with tacrolimus produced additive effects in liver reperfusion-injury of rat, although treatment with ONO-4057 alone had no effect.

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COMMENTS

Background

Ischemia-reperfusion injury is a major factor that might influence early graft function in liver transplantation. In this study, we investigated the effect of oral administration, a novel Leukotriene B₄ (LTB₄) receptor antagonist, and/or Tacrolimus on ischemia-reperfusion of rat liver model.

Research frontiers

In ischemia-reperfusion injury of the liver, an excessive inflammatory response is considered as a key mechanism. The role of neutrophils in ischemia-reperfusion injury has recently been the focus of several investigations. In this study, we focused the importance of neutrophils.

Innovations and breakthroughs

We tested the combined effect of a LTB₄ receptor antagonist with Tacrolimus for ischemia-reperfusion injury of the liver.

Applications

The findings in this study support the hypothesis that co-administration of ONO-4057 and tacrolimus might be reasonable for liver transplantation in the future.

Peer review

This manuscript is sufficiently written and provides information concerning therapeutic options in I/R injury of the liver.

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BRIEF ARTICLE

Glutathione-S-transferase (GSTM1, GSTT1) and the risk of gastrointestinal cancer in a Korean population

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Author contributions: Piao JM designed the study, performed the majority of experiments, data analysis and interpretation and wrote the manuscript; Kim HN provided vital reagents and analytical tools and was also involved in editing the manuscript; Choi JS, Kweon SS and Kim SH provided the collection of all the DNA material and control sample; Bae WK, Shim HJ, Kim HR and Park YK collected clinical data; Choi YD collected clinicopathological data, Shin MH interpreted study findings, supervised the study and approved the final version of the manuscript.

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of GSTM1 and GSTT1 were determined using real-time PCR.

RESULTS: The null genotypes of GSTM1 and GSTT1 were not significantly associated with elevated risk of gastric (OR = 1.070, 95% CI = 0.935-1.224; OR = 1.101, 95% CI = 0.963-1.259, respectively) or colorectal cancer (OR = 1.065, 95% CI = 0.923-1.228; OR = 1.041, 95% CI = 0.903-1.200, respectively). The frequency of the combined null GST genotype was not different between the two cancer groups and controls. Moreover, smoking, drinking, and age did not modify the association between these genotypes and the risk of gastric or colorectal cancer.

CONCLUSION: GSTM1 and GSTT1 null genotypes were not associated with increased risk of GC or CRC in Koreans.

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Key words: Glutathione S-transferase mu; Glutathione S-transferase theta; Gastric cancer; Colorectal cancer; South Korean population

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Abstract

AIM: To evaluate the association of glutathione S-transferase mu (GSTM1) and glutathione S-transferase theta (GSTT1) null genotypes with the risk of gastric cancer (GC) and colorectal cancer (CRC) in a South Korean population.

METHODS: We conducted a population-based, large-scale case-control study including 2213 GCs, 1829 CRCs, and 1699 controls. Null and non-null genotypes

INTRODUCTION

Gastric cancer (GC) and colorectal cancer (CRC) are the most common malignancies in Korea. Environmental factors, such as diet, infection, and smoking, and genetic factors have been shown to play a role in the development of these malignancies^[1-4].

The glutathione S-transferase (GST) enzymes

are involved in detoxification of many potentially carcinogenic compounds. The enzymes are encoded by at least five distantly related gene families (the alpha, mu, pi, sigma, and theta GSTs). In humans, marked interindividual differences exist in the expression of mu (GSTM1), and theta (GSTT1) GSTs^[5]. The GSTM1 and GSTT1 null genotypes have been linked to increased risk of developing lung, bladder, colon, and skin cancers^[5-7], and several studies have shown that GSTM1 and GSTT1 null genotypes were associated with increased risk of GC^[8,9] and CRC^[10]. However, some data have suggested that no relationship exists between the GSTM1 or GSTT1 null genotype and the risk of GC^[11,12] or CRC^[13,14]. The contradictory results regarding the association between GSTM1 and GSTT1 null genotypes and the risk of GC or CRC may be due to limited sample sizes or differences in the ethnicities or differences in the genetic subtypes studied, or they may also be attributable to differences in exposure to environmental factors. However, the majority of the reports involved small sample sizes, so the association of the GSTM1/GSTT1 null genotype and the risk of GC and CRC need to be confirmed in studies with larger numbers of samples.

The present study aimed to evaluate the association of the GSTM1 and GSTT1 null genotypes with the risk of GC and CRC, and to determine whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk.

MATERIALS AND METHODS

Ethics

This study was approved by the Institutional Review Board of the Chonnam National University Hwasun Hospital in Hwasun, Korea, and all patients provided informed written consent.

Subjects

The study included 4042 newly diagnosed cancer cases (2213 GC and 1829 CRC) and 1699 controls. The cases were histologically confirmed at the Chonnam National University Hwasun Hospital (Jeollanam-do, Korea), between April 2004 and June 2008. Cases with secondary or recurrent tumors were excluded. The tumor stages were classified according to the TNM classification, including clinical or pathological TNM stages. GC was classified by anatomical site as cardia (C16.0) or non-cardia (C16.1-16.8) and by histological type as intestinal, diffuse, or mixed type.

The control group ($n = 1699$) consisted of participants in the Thyroid Disease Prevalence Study conducted from July 2004 to January 2006 in Yeonggwang and Muan Counties of Jeollanam-do Province and in Namwon City of Jeollabuk-do, Korea^[15]. At the time of peripheral blood collection, all case and control subjects provided their informed consent to participate in this study.

Blood samples and DNA isolation

Blood samples were collected in EDTA-containing tubes, and DNA was extracted from the buffy coat for genotyping. Genomic DNA was extracted using a

QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Genotyping

GSTM1 and GSTT1 genotyping was performed using a TaqMan allelic discrimination assay with previously described primers and modified probes^[16]. Real-time PCR was performed using a Rotor-Gene 3000 multiplex system (Corbett Research, Sydney, Australia) in a 10- μ L reaction volume containing 200 nmol/L PCR primer, 10 nmol/L CY5-labeled probe for GSTT1, 100 nmol/L FAM-labeled probe for GSTM1, 0.5 U of *f-Taq* polymerase (Solgent, Daejeon, Korea), and 40 ng of genomic DNA. The primer and probe are as follows: GSTM1, Forward, 5'-GGAGACAGAAGAGGAGAAGATTTC-3', Reverse, 5'-GCCCAGCTGCATATGGTTGT-3', Probe, FAM-CCATGGTCTGGTTCTCCAAAATGTCCA-BHQ1; GSTT1, Forward, 5'-CTTCCAGGAGGCCCATGAG-3' Reverse, 5'-CAGGGCATCAGCTTCTGCTT-3', Probe, CY5-AAGGACTTCCCACCTGCAGACCCC-BHQ3.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0. The descriptive data for the major characteristics of study groups are expressed as mean (range) and percent. We used *t*-tests to determine statistical differences in the continuous variables and χ^2 tests for the categorical variables. Adjusted odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated using logistic regression models with adjustments for age and sex to estimate the association between genotype and GC or CRC. Interactions of genotype with smoking, alcohol consumption, and age were estimated using the logistic regression model, which included an interaction term as well as variables for exposure (smoking and alcohol drinking), genotypes, and potential confounders (sex and age). Subjects with wild-type genotypes were considered to have baseline risk. The subjects for which there was missing data for smoking, drinking, anatomical site, and histological type TNM staging were excluded in interaction analysis related with these variables. All tests were conducted at the $P = 0.05$ level of significance.

RESULTS

We included 2213 cases of GC, 1829 cases of CRC, and 1699 cancer-free controls in the present study. The demographic characteristics of subjects are shown in Table 1. The proportion of men in the cancer cases was higher than that in the controls, and cases in both cancer groups tended to be older than controls. The proportion of smoking in GC cases was higher than that in the controls, but in CRC cases was lower than that in the controls. The proportion of drinking in both cancer groups was lower than that in the controls.

The frequency distributions of the GSTM1 null genotype in the control, GC, and CRC groups were 54.3%, 55.4%, and 54.9%, respectively. The frequency distributions of the GSTT1 null genotype in the control, GC, and CRC groups were 50.5%, 53.0%, and 51.9%,

Table 1 Demographic characteristics of subjects *n* (%)

Characteristics	Controls	GC	CRC
No.	1699	2213	1829
Age, mean (yr, range)	52.2 (20-74)	60.2 (21-87) ^a	61.9 (22-88) ^c
≤ 65 yr	1320 (77.7)	1314 (59.4)	985 (53.9)
> 65 yr	379 (22.3)	899 (40.6) ^a	844 (46.1) ^c
Sex			
Male	820 (48.3)	1510 (68.2)	1149 (62.8)
Female	879 (51.7)	703 (31.8) ^a	680 (37.2) ^c
Smoking			
Never-smokers	1000 (58.8)	1127 (50.9)	1137 (62.2)
Ever-smokers	654 (38.5)	997 (45.1) ^a	582 (31.8) ^c
Missing	45 (2.6)	89 (4.0)	110 (6.0)
Drinking			
Never drinkers	825 (48.6)	1198 (54.1)	1084 (59.3)
Current drinkers	832 (49.0)	921 (41.6) ^a	623 (34.1) ^c
Missing	42 (2.5)	94 (4.3)	122 (6.7)
GSTT1			
Null type	858 (50.5)	1172 (53.0)	950 (51.9)
Wild type	841 (49.5)	1041 (47.0)	879 (48.1)
GSTM1			
Null type	923 (54.3)	1225 (55.4)	1004 (54.9)
Wild type	776 (45.7)	988 (44.6)	825 (45.1)
TNM stage			
I		1138 (51.4)	291 (15.9)
II		305 (13.8)	547 (29.9)
III		290 (13.1)	615 (33.6)
IV		386 (17.4)	230 (12.6)
Unspecified stage		94 (4.2)	147 (8.0)
Tumor site			
Cardiac		106 (4.8)	833 (45.5) (colon)
Non-cardiac		2093 (94.6)	996 (54.5) (rectum)
Unspecified site		14 (0.6)	
Histological type			
Intestinal		1286 (58.1)	
Diffuse		561 (25.4)	
Mixed		240 (10.8)	
Unspecified type		126 (5.7)	

^aGC compared with control, $P < 0.05$; ^cCRC compared with control, $P < 0.05$. GC: Gastric cancer; CRC: Colorectal cancer; GSTT1: Glutathione S-transferase theta; GSTM1: Glutathione S-transferase mu.

respectively. No significant differences were observed in the frequencies of the GSTT1 and GSTM1 genotypes between cancer patients and controls (Table 1).

The GSTM1 and GSTT1 null genotypes were not significantly associated with risk of GC (OR = 1.070, 95% CI = 0.935-1.224; OR = 1.101, 95% CI = 0.963-1.259, respectively) or CRC (OR = 1.065, 95% CI = 0.923-1.228; OR = 1.041, 95% CI = 0.903-1.200, respectively). The GSTM1 and GSTT1 null genotypes were not significantly associated with the risk of GC or CRC, classified according to TNM stage, tumor site or histology type (GC) (Tables 2 and 3).

Smoking, alcohol consumption and age did not modify the association between the GSTM1 and GSTT1 null genotypes and the risk of GC or CRC (Tables 2 and 3).

No difference in the frequency of the combined GSTM1 and GSTT1 null genotype was observed between the two cancer groups and controls (Table 4).

DISCUSSION

The present large-scale study investigated the association

Table 2 GST (M1, T1) genotypes in relation of GC risk stratified by some factors

	GSTM1 (null)		GSTT1 (null)	
	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^a
All case	1.070 (0.935-1.224)		1.101 (0.963-1.259)	
Smoking				
Never smoker	0.994 (0.809-1.222)		1.099 (0.895-1.350)	
Ever smoker	1.127 (0.938-1.355)	0.385	1.082 (0.901-1.299)	0.921
Drinking				
Never drinkers	1.103 (0.916-1.328)		1.116 (0.927-1.344)	
Current drinkers	1.019 (0.829-1.253)	0.559	1.077 (0.877-1.322)	0.748
Age (yr)				
≤ 65 yr	1.069 (0.910-1.256)		1.129 (0.961-1.325)	
> 65 yr	1.053 (0.823-1.348)	0.990	1.056 (0.825-1.352)	0.621
TNM stage				
I + II	1.045 (0.900-1.214)		1.130 (0.974-1.312)	
III+IV	1.050 (0.870-1.267)		1.044 (0.865-1.259)	
Tumor site				
Cardiac	1.200 (0.793-1.817)		1.030 (0.684-1.553)	
Non-cardiac	1.060 (0.925-1.215)		1.100 (0.961-1.260)	
Histological type				
Intestinal	1.050 (0.892-1.235)		1.158 (0.984-1.362)	
Diffuse	1.007 (0.830-1.220)		1.055 (0.871-1.278)	
Mixed	1.210 (0.910-1.609)		0.975 (0.736-1.292)	

Adjusted for age, sex; *P*^a for *P* interaction.

Table 3 GST (M1, T1) genotypes in relation of CRC risk stratified by some factors

	GSTM1 (null)		GSTT1 (null)	
	OR (95% CI)	<i>P</i> ^a	OR (95% CI)	<i>P</i> ^a
All case	1.065 (0.923-1.228)		1.041 (0.903-1.200)	
Smoking				
Never smoker	1.040 (0.822-1.316)		0.999 (0.790-1.262)	
Ever smoker	1.077 (0.889-1.304)	0.907	1.067 (0.882-1.291)	0.659
Drinking				
Never drinkers	1.025 (0.845-1.243)		1.052 (0.868-1.275)	
Current drinkers	1.170 (0.928-1.475)	0.426	1.084 (0.862-1.364)	0.870
Age (yr)				
≤ 65	1.061 (0.890-1.265)		1.054 (0.885-1.256)	
> 65	1.070 (0.835-1.370)	0.999	1.039 (0.811-1.330)	0.901
TNM stage				
0+ I + II	1.054 (0.882-1.261)		1.106 (0.926-1.323)	
III+IV	1.101 (0.924-1.312)		1.035 (0.870-1.232)	
Tumor site				
Colon	0.980 (0.822-1.169)		1.090 (0.914-1.300)	
Rectum	1.149 (0.970-1.360)		0.996 (0.842-1.178)	

Adjusted for age, sex; *P*^a for *P* interaction.

between GSTM1 and GSTT1 null genotypes and susceptibility to GC and CRC in a South Korean population. In this study, we observed no significant association between either type of cancer and the GSTM1 and GSTT1 null genotypes. Additionally, no difference was observed in the frequency of the combined GST (M1 and T1) null genotype between the two cancer groups and controls. Moreover, smoking did not modify the association between these polymorphisms and risk of GC or CRC.

The reports examining the GSTM1 and GSTT1 null genotypes and their association with gastric cancer are quite inconsistent. Two reports suggested that the GSTM1

Table 4 Combined GSTT1/GSTM1 genotype frequencies and their associations with risk GC, CRC *n* (%)

Combined genotypes	GC	CRC	Controls	OR ^{1a} (95% CI)	OR ^{1b} (95% CI)
T1(+)/M1(+)	607 (22.1)	478 (21.4)	385 (22.7)	1	1
T1(+)/M1(-)	707 (25.7)	583 (26.1)	456 (26.8)	1.02 (0.84-1.23)	1.19 (0.97-1.47)
T1(-)/M1(+)	632 (23.0)	523 (23.4)	391 (23.0)	1.04 (0.86-1.27)	0.97 (0.79-1.19)
T1(-)/M1(-)	800 (29.1)	648 (29.0)	467 (27.5)	1.17 (0.97-1.41)	1.11 (0.91-1.36)

Adjusted for age, sex; OR^{1a} for gastric cancer; OR^{1b} for colorectal cancer.

null genotype increased GC risk^[8,10], and three reported that the GSTT1 null genotype increased GC risk^[9,17,18]. However, our results suggest that no associations exist which is in line with the majority of reports^[11,12,19-21]. In studies of Korean populations, no significant associations were detected between the GSTM1 and GSTT1 null genotypes and GC risk. One study conducted in Iksan, Korea, reported that the GSTM1 and GSTT1 null genotypes had no association with the risk of GC (for GSTM1 null, OR = 0.86 and 95% CI = 0.49-1.51; for GSTT1 null, OR = 0.97 and 95% CI = 0.55-1.71)^[12]. Hong *et al.*^[20] reported similar results. Another study suggested that the GSTM1 and GSTT1 null genotypes were not associated with the risk of GC overall, but that in individuals who consumed kimchi, a spicy Korean food made with fermented cabbage, the GSTM1 and GSTT1 non-null genotype increased the risk of GC^[19]. Two meta-analyses, by La Torre *et al.*^[22] and Boccia *et al.*^[23] suggested that the GSTM1 and GSTT1 null genotypes have no effect on the risk of GC per se, but may modulate tobacco-related carcinogenesis of gastric cancer.

Two studies reported that the GSTT1 null genotype increased the risk of CRC^[10,24], and three studies reported decreased CRC risk, one for the GSTM1 null^[25] and two for the GSTT1 null genotype^[26,27]. However, five studies suggested that the GSTM1 and GSTT1 null genotype are not related to CRC risk^[4,8,13,14,28]. One Korean study suggested that the genotypes of GSTM1 are associated with cancer occurrence in individuals carrying the hMLH1/hMSH2 mutation who were family members of patients with hereditary nonpolyposis colorectal cancer^[29].

The conflicting results regarding the associations between GSTM1 and GSTT1 null genotypes and risks for GC and CRC may be due to limited sample size or differences in the ethnicities or genetic subtypes studied, and they may also be attributable to differences in exposure to environmental factors.

Polycyclic aromatic hydrocarbons (PAHs) are the main carcinogens in tobacco smoke. The ultimate carcinogen (PAH-DE) can be detoxified through conjugation with glutathione by GSTs, which are phase II enzymes^[30]. Individuals with the null genotype of GSTM1 or GSTT1 would have less capacity for detoxification of PAHs, which would potentially increase their risk of chemical carcinogenesis. However, our data suggested that smoking did not modify the association between the GSTM1 and GSTT1 null genotypes and the risk of GC or CRC.

Four studies examined the interaction between smoking, GSTM1 or GSTT1 polymorphisms, and the risk of GC^[10,11,18,21]. One study suggested that smoking modi-

fies the association between the GSTM1 null genotype and the risk of GC^[21]. In contrast, three studies reported that smoking did not modify the association between the GSTM1 and/or GSTT1 null genotype and GC risk^[10,11,18], in line with our results. Additionally, six studies evaluated the interaction between smoking, the GSTM1 or GSTT1 null genotype, and the risk of CRC^[13,25,31-34]. Two studies reported that smoking modifies the association between the GSTM1 and/or GSTT1 null genotype and CRC risk^[13,31]. However, four studies reported no interaction between the GSTM1 and/or GSTT1 null genotype and smoking in CRC risk^[25,32-34], in agreement with our results.

Our study suggests that alcohol consumption did not modify the association of GSTM1 and GSTT1 null genotypes with the risk of GC or CRC, which is consistent with previous studies regarding GC^[9,11,18]. However, no studies have been reported examining whether alcohol consumption modifies the association of the GSTM1 or GSTT1 null genotype with the risk of CRC.

The detoxification potential of the GSTs was observed to decrease with age^[35]. Yeh *et al.*^[24] reported that men aged ≤ 60 years with the GSTT1 null genotype were at significantly increased risk of rectal cancer, and one study reported the GSTT1 null genotype was not associated with the risk of GC, classified according to age less than or greater than 60 years^[17]. However, the present study suggested that age does not modify the association between GSTM1 and GSTT1 null genotypes and the risk of GC or CRC.

In our data, the GSTM1 and GSTT1 null genotypes were not significantly associated with the risk of GC or CRC, classified according to TNM stage, tumor site, and histology type (GC). To the best of our knowledge, no reports have been published regarding the GSTM1 and GSTT1 null genotypes and GC or CRC risk classified according to TNM stage.

Two studies investigated the association between the GSTM1 and GSTT1 null genotypes and the risk of cardia or non-cardia GC, and both studies suggested that no significant association exists^[3,36], in line with our result. Seow *et al.*^[37] reported that the GSTM1 and GSTT1 null genotypes were not associated with the risk of CRC, classified according to location of the tumor in the colon or the rectum. Suzuki *et al.*^[38] reported finding no association between the GSTM1 null genotype and the risk of intestinal type or diffuse type GC, and Agudo *et al.*^[3] reported finding no association between the GSTT1 null genotype and the risk of intestinal type or diffuse type GC, consistent with our results.

In our data, smoking was associated with increased risk

of GC, but with decreased risk of CRC. Previous studies reported that smoking increased the risk of GC^[39], in line with our results. The association between smoking and CRC has been inconsistent among studies. A recent meta-analysis suggested that smoking is significantly associated with CRC incidence^[40]. In our data, drinking was associated with decreased risk of both cancer groups. Drinking probably does not affect overall risk of stomach cancer, but there is some evidence that drinking may increase the risk of GC^[41]. The relationship between drinking and CRC risk has been controversial, but a meta-analysis found that high consumers of alcohol had an elevated CRC risk^[42]. We could not rule out the possibility that differential misclassification bias may have occurred in our study, because we retrospectively gathered information about smoking and drinking from electronic medical records in both case groups, while cross-sectional surveys were used to gather information about smoking and alcohol in controls.

The major strength of our study was its large sample size. Ours was the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population.

The limitations of our study must also be acknowledged. First, the study did not consider genetic polymorphisms of other cancer related genes. Second, the study considered a limited number of environmental factors (smoking and alcohol consumption), and other environmental factors such as dietary intake were not considered.

In conclusion, the results of the present population-based, large-scale case-control study suggest that in a Korean population, the GSTM1/GSTT1 null genotype does not modulate an individual's susceptibility to GC or CRC, and that smoking, alcohol consumption, and age do not modify the association between these genotypes and the risk of GC or CRC.

COMMENTS

Background

Gastric cancer (GC) and colorectal cancer (CRC) are the most common malignancies in Korea. Environmental factors and genetic factors have been shown to play a role in the development of these malignancies.

Research frontiers

The glutathione S-transferase enzymes are involved in detoxification of many potentially carcinogenic compounds. Several studies have shown that glutathione S-transferase mu (GSTM1) and glutathione S-transferase theta (GSTT1) null genotypes were associated with increased risks for GC and CRC. But some data suggested no relationship between GSTM1/GSTT1 null genotype and the risk of GC and CRC.

Innovations and breakthroughs

This is the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population. It also aimed to determine whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk.

Applications

This study suggested that GSTM1 and GSTT1 null genotypes were not associated with increased risk of GC or CRC in Koreans. Smoking, drinking, and age did not modify the association between these genotypes and the risk of gastric or colorectal cancer. Future research should focus on other parts of the GST genotype to understand its role and risk of gastric and colorectal cancer in Korean population.

Terminology

GST: Glutathione-S-transferase is a Phase II detoxification enzyme. The

enzymes are encoded by at least five distantly related gene families (the alpha, mu, pi, sigma, and theta GSTs). In humans, marked interindividual differences exist in the expression of mu (GSTM1), and theta (GSTT1) GSTs.

Peer review

This is the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population. It also determined whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk. Although results of this study show no association between the null genotypes of GSTM1 and GSTT1 and risk of gastric and colorectal cancer, and no-association results are not as attractive as positive associations, I feel they should be presented to the scientific audience in order not to create a literature bias towards publishing only studies with positive associations.

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BRIEF ARTICLE

Gastric dysplasia may be an independent risk factor of an advanced colorectal neoplasm

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CONCLUSION: The study emphasizes the need for colon surveillance in patients with gastric dysplasia, regardless of *H pylori* infection.

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Key words: Gastric adenoma or dysplasia; *Helicobacter pylori*; Colorectal neoplasm

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Abstract

AIM: To evaluate the relationship between gastric dysplasia and *Helicobacter pylori* (*H pylori*) and the occurrence of colorectal adenoma, and to define the necessity for colonoscopy in patients with gastric dysplasia or *H pylori* infection.

METHODS: From May 2005 to February 2008, 133 patients with established gastric dysplasia by gastroduodenoscopy (EGD) were additionally investigated by colonoscopy. The authors compared results with those of 213 subjects who underwent both EGD and colonoscopy during the same period at the author's Health Promotion Center as a control group. *H pylori* infection was evaluated in both the gastric dysplasia and control groups.

RESULTS: The mean age of all 346 study subjects was 54.1 ± 10.5 years, and there were 258 (73%) men and 87 (27%) women. No significant difference was found between the *H pylori* positive and negative subjects in terms of the prevalence of colorectal adenoma and advanced colorectal adenoma ($P = 0.261$). Patients with gastric dysplasia showed no elevated risk of colorectal adenoma (OR = 0.910, 95% CI: 0.587-1.411, $P = 0.738$), but had a significantly higher risk of having advanced colorectal adenoma (OR = 3.382, 95% CI: 1.700-6.342, $P = 0.000$).

INTRODUCTION

Colorectal carcinoma is one of the most common cancers and is the second leading cause of cancer-related mortality in the United States^[1], and the fourth leading cause of cancer-related mortality in Korea^[2]. The etiologies and risk factors of colorectal neoplasms have attracted research attention for several decades, especially in association with *Helicobacter pylori* (*H pylori*) or gastric dysplasia. *H pylori* infection is a well-known cause of chronic gastritis, peptic ulcers^[3,4], gastric adenocarcinoma^[5], and gastric lymphoma^[6], and several reports have suggested that *H pylori* infection increases the risk of colorectal adenoma and adenocarcinoma^[7-9]; however, other reports disagree^[10,11]. Furthermore, several have concluded that gastric adenoma and carcinoma may be indicators of colorectal adenoma or carcinoma^[12,13], and it is accepted that both gastric adenoma and colorectal adenoma have a high potential for malignant transformation^[14-16]. The aims of this study were to evaluate relations between gastric dysplasia and *H pylori* infection and the occurrence of colorectal adenoma and to define the need for colonoscopy in patients with gastric dysplasia or *H pylori* infection.

MATERIALS AND METHODS

Patients

From May 2005 to February 2008, 133 consecutive patients with established gastric dysplasia (the gastric dysplasia group), as determined by gastroduodenoscopy (EGD) at our institute, were additionally subjected to colonoscopy, after obtaining informed consent, within 3 wk of EGD. We defined gastric adenoma as gastric dysplasia. The presence of gastric dysplasia and colorectal adenoma were confirmed histologically using specimens obtained by endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), or polypectomy. We also enrolled 213 consecutive subjects who underwent both EGD and colonoscopy for the purpose of screening at the Health Promotion Center at Kyungpook National University Hospital as a control group during the same period. We reviewed endoscopic findings and pathology specimens retrospectively. The subjects who were included in the control group had no symptoms and no gastric dysplasia. Patients with any kind of malignancy and those with a previous history of colorectal surgery were excluded. Subjects with either family history of colorectal adenocarcinoma or adenoma or gastric cancer were also excluded. This study was approved by our Institutional Review Board.

Diagnosis of *H pylori* infection

H pylori infection was diagnosed by biopsy, CLO test (GASTREX, Warsaw, Poland), or by using an urea breathing test (UBT, Otsuka, Tokyo). The specimens for the CLO test were obtained from both the antrum and the distal body of the stomach during EGD. Cresyl violet staining was used for the pathologic diagnosis of *H pylori* infection. The cut-off value of the UBT was 2.5‰. *H pylori* infection was diagnosed when two of the above three methods were positive.

Colonoscopic diagnosis

Cecal intubation was successful in all patients. All lesions found during colonoscopy were removed by biopsy, polypectomy, EMR, or ESD. Advanced colorectal adenoma was defined as a size larger than 1 cm, a villous or tubulovillous adenoma histology, or adenoma with high-grade dysplasia.

Statistical analysis

We compared age and sex distributions, the presence or absence of colorectal adenoma, colorectal adenoma sizes and histology, and *H pylori* infection states in the gastric dysplasia and control groups. Variables were compared using the χ^2 or independent *t*-tests, depending on the nature of the data. Fisher's exact test was used when numbers were small. Odds ratios (ORs) and 95% CIs were used to describe associations. ORs with corresponding 95% CIs were obtained by conditional logistic regression analysis. *P* values of < 0.05 were considered statistically significant, and statistical calculations were performed using SPSS version 14.0 software for Window (SPSS Inc. Chicago, IL, USA).

Table 1 Baseline characteristics of gastric dysplasia and control group

	Gastric dysplasia (<i>n</i> = 133)	Control (<i>n</i> = 213)	<i>P</i> -value
Age (mean $\pm$ SD)	61.2 $\pm$ 9.7	49.6 $\pm$ 8.3	0.0005
Sex [M/F, (%)]	97/36 (73/37)	162/51 (76/24)	0.5200
<i>H pylori</i>			0.2610
(+) <i>n</i> = 204	73	131	
(-) <i>n</i> = 142	60	82	
Colorectal adenoma			0.5220
(+) <i>n</i> = 148	55	93	
(-) <i>n</i> = 198	78	120	
Advanced adenoma			0.0007
(+) <i>n</i> = 44	28	16	
(-) <i>n</i> = 302	105	197	

Advanced adenoma: Advanced colorectal adenoma.

RESULTS

Clinical characteristics of the gastric dysplasia and control groups

The mean age of all 346 study subjects was 54.1 ± 10.5 years, and there were 258 men (73%) and 87 women (27%). The gastric dysplasia group was older than the control group ($P = 0.0005$). In the gastric dysplasia group ($n = 133$), colorectal adenoma was diagnosed in 55/133 (41%) and advanced colorectal adenoma in 28/133 (21%). In the control group, colorectal adenoma was diagnosed in 93/213 (44%), and advanced colorectal adenoma in 16/213 (7.5%). No significant difference was observed between the gastric dysplasia and control groups in terms of the prevalence of colorectal adenoma ($P = 0.5220$), and no significant difference was observed between the *H pylori* positive and negative groups in terms of the prevalence of gastric dysplasia, colorectal adenoma, or advanced colorectal adenoma ($P = 0.2610$). Furthermore, no significant difference was observed between the gastric dysplasia and control groups in terms of the sizes of advanced colorectal adenomas (10.50 ± 3.107 mm *vs* 11.92 ± 8.722 mm, respectively, $P = 0.5860$) (Table 1).

Clinical characteristics of the advanced colorectal adenoma positive and negative groups

Mean age in the advanced colorectal adenoma positive group was greater than in the negative group ($P = 0.0002$). However, group sex distributions ($P = 0.0600$) and *H pylori* ($P = 0.1060$) infection statuses were not different significantly different (Table 2).

Comparisons of the colorectal adenoma and advanced colorectal adenoma groups

Those with colorectal adenoma or advanced colorectal adenoma were older than those without (Table 3). Moreover, those with gastric dysplasia showed no increased risk of having colorectal adenoma (OR = 0.910, 95% CI: 0.587-1.411, $P = 0.7380$), but they had a significantly higher risk of having advanced colorectal adenoma (adjusted OR = 3.283, CI: 1.700-6.342, $P = 0.0004$) (Table 3). This result was supported by multivariate analysis (Table 4).

Table 2 Baseline characteristics of advanced adenoma (+) and (-) group

	Advanced adenoma (+) (n = 44)	Advanced adenoma (-) (n = 302)	P-value
Age (mean ± SD)	60.8837 ± 8.55294	53.1419 ± 10.43410	0.0002
Sex [M/F n (%)]	38/6 (86/14)	221/81 (73/27)	0.0600
<i>H pylori</i>			0.1060
(+) n = 204	21	183	
(-) n = 142	23	119	

DISCUSSION

This study shows that the risk of advanced colorectal adenoma is elevated in patients with gastric dysplasia, and thus, supports the notion that colorectal cancer risk is increased by gastric adenoma, as has been reported previously^[12]. In addition our study concurs with the findings of a previous study, in which it was found that two of six patients with diffuse gastric adenoma had colon polyps and 40 of 73 patients (54.8%) with gastric polyps had colorectal adenomatous polyps^[17]. In the present study, 55 of 133 patients (41%) with gastric dysplasia had colorectal adenomatous polyps and 28 of 133 (21%) had advanced colorectal adenomatous polyps. The present study concurs with another study, in which it was found that eight of eleven patients with duodenal adenomas had colonic polyps^[18]. Furthermore, the present study agrees with other studies concerning the elevated risk of gastric polyps in patients with sporadic colonic polyps^[19]. Shemesh *et al*^[20] reported that two of 100 patients with 1-4 colorectal polyps had gastric adenoma, 3 of 80 patients (3.5%) with 5 or more colorectal polyps had gastric adenoma, 3 of 80 patients with colon cancer had gastric adenoma, and one of 100 patients without colorectal adenoma and cancer had gastric adenoma, and these findings are also in line with those of the present study. The progression of colorectal adenoma to colon cancer is known to be associated with an adenoma size larger than 10 mm, more than three lesions, a villous, tubulovillous or high grade dysplastic histology, and the presence of genetic abnormalities^[21-24]. Genetic abnormalities in colon cancer are very similar to those in gastric cancer^[25,26], especially concerning mutations of the *p53*^[27,28], *APC*^[29,30], *DCC*^[31,32], and *K-ras* genes^[33,34]. Genetic abnormalities are well known to be associated with upper gastrointestinal polyps and with hereditary colonic polyposis syndromes^[25,26], which include familial polyposis coli^[25,26], Gardner's syndrome, Peutz-Jeghers' syndrome, Cowden's syndrome, Cronkhite-Canada syndrome, hereditary flat adenoma syndrome, and others. In the present study, we deliberately excluded patients with HNPCC, suspected HNPCC, or FAP to avoid the influence of such genetic factors.

Gastric adenoma or dysplasia have high rates of malignant transformation (up to 75%^[14-16,19-21]). Accordingly, regardless of the existence of colorectal adenoma, treatment of gastric dysplasia by EMR or ESD is inevitable. Moreover, the above-mentioned relations suggest that the pathogeneses of gastric dysplasia and colorectal

Table 3 Statistics of colorectal adenoma and advanced adenoma groups

	Colorectal adenoma			Advanced adenoma		
	aOR	CI	P-value	aOR	CI	P-value
Age (old age)	1.337	0.872-2.049	0.0020	2.872	1.446-5.704	0.0002
Sex (female)	0.629	0.379-1.042	0.0800	0.431	0.176-1.057	0.0640
<i>H pylori</i>	1.037	0.672-1.599	0.9120	0.594	0.315-1.120	0.1390
(positive)						
G. dysplasia	0.910	0.587-1.411	0.7380	3.283	1.700-6.342	0.0004
(presence)						

aOR: Adjusted odds ratio; G. dysplasia: Gastric dysplasia.

adenoma are similar. Several other controversial factors may increase the risk of colorectal cancer development, e.g. an increased serum gastrin level^[35], *H pylori* infection^[6-11] and *Streptococcus bovis* bacteremia^[36]. However, previous studies have concluded that the presence of *H pylori* infection and an elevated serum gastrin level are not associated with elevated risks of colorectal neoplasm development. Recently, some studies have concluded that increased serum gastrin levels due to the use of proton pump inhibitors do not predispose the development of a colorectal neoplasm. In the present study, we included only gastric dysplasia and *H pylori* infection status to examine the risk of colorectal adenoma development. No significant association was found between *H pylori* infection and colorectal adenoma, which concurs with another study^[10,11], and thus, it appears that an agent other than *H pylori* is responsible for co-pathogenesis of colorectal adenoma^[35].

The aim of several studies on colorectal adenoma has been to reduce colorectal cancer mortality and morbidity. To achieve this aim, the early diagnoses and treatment of precancerous lesions, like colorectal adenoma and early colorectal cancer, are of the utmost importance, especially, in those deemed to be at high risk group by early surveillance colonoscopy^[37,38]. Some studies have recommended that gastric cancer patients undergo colonoscopy due to a substantial risk of their developing colon cancer^[13]. In another study, haemoccult studies were recommended for early detection of colorectal tumors in patients with gastric polyp^[39]. In addition, it has been reported that the rates of occurrence of colorectal adenoma in gastric adenoma and carcinoma patients are increasing^[12]. In common with other studies, the present study reveals that gastric dysplasia is an indicator of advanced colorectal adenoma, which is an important risk factor of colorectal cancer. Accordingly, we emphasize the need for colon surveillance in elderly patients with gastric dysplasia because of their elevated risk of developing advanced colorectal adenoma, which is an important risk factor of colorectal cancer, regardless of *H pylori* infection.

Several limitations of the present study should be considered. First, the sample size was small, which introduces the possibility of a type II error, and we could not collect age and sex-matched controls. Second, selection bias is possible due to the retrospective nature of the study. Third, insufficient information was obtained regarding alcohol consumption, smoking status, and

Table 4 Multivariate analysis of colorectal adenoma and advanced adenoma groups

	Colorectal adenoma					Advanced colorectal adenoma				
	B	SE	t	Sig	95% CI	B	SE	t	Sig	95% CI
Age	3.585	1.129	3.176	0.0020	1.365 to 5.805	7.770	1.648	4.714	0.0002	4.529 to 11.01
Sex	-0.085	0.047	-1.810	0.0710	-0.007 to 0.178	-0.132	0.070	1.888	0.0600	-0.269 to -0.006
<i>H pylori</i>	-0.009	0.054	-0.163	0.8710	-0.114 to 0.097	-0.129	0.079	1.623	0.1060	-0.285 to -0.027
G. dysplasia	0.022	0.053	0.421	0.6740	-0.082 to 0.127	0.289	0.077	3.741	0.0004	0.137 to 0.440

serum gastrin, cholesterol, and glucose levels, which were recently proposed as risk factors of gastric dysplasia^[35]. Fourth, the mean age of the gastric dysplasia group was greater than that of the control group, because the patients who visited our health promotion center were much younger than the gastric dysplasia patients. Accordingly, although age-adjusted odds ratios for advanced colorectal adenoma were statistically significance, we cannot exclude the possibility of an age-related bias.

Even though there were several reports that *H pylori* infection increased the risk of colorectal adenoma and adenocarcinoma^[7-9], our report reveals that *H pylori* infection is not a risk factor for advanced colorectal polyps, but that gastric dysplasia and an advanced age are. Thus, we infer that genetic and environmental factors probably importantly contribute to the development of advanced colorectal polyps, because patients are continuously exposed to those factors. However, the environmental factors involved have not been identified as yet. Thus, further diverse complex study models are required to clarify the relationship between gastric dysplasia, *H pylori*, and colorectal adenoma, and identify the environmental factors concerned. Moreover, a large prospective case-controlled study is required to determine whether the presence of gastric dysplasia is an indication for colonoscopy and what intervals would be recommended for patients with gastric dysplasia.

COMMENTS

Background

Over the last decades, several studies have been performed on the etiologies and risk factors of colorectal neoplasms, especially on the association with *Helicobacter pylori* (*H pylori*) and gastric dysplasia. This study was performed to define the necessity of screening colonoscopy in patients with gastric dysplasia or *H pylori* infection.

Research frontiers

Several controversial factors might increase the risk of colorectal cancer development, for example, increased serum gastrin level, *H pylori* infection and *Streptococcus bovis* bacteremia, and so on. In this study, the authors demonstrate the relations between gastric dysplasia and the occurrence of advanced colorectal adenoma.

Innovations and breakthroughs

Even though there were several reports that *H pylori* infection increased the risk of colorectal adenoma and adenocarcinoma, this report reveals that *H pylori* infection is not a risk factor for advanced colorectal polyps; however, gastric dysplasia and an advanced age are. Thus, genetic and environmental factors probably contribute to the development of advanced colorectal polyps, because of continuous exposure to genetic and environmental factors.

Applications

This result could emphasize the need for colon surveillance in elderly patients with gastric dysplasia because of their elevated risk of developing advanced

colorectal adenoma, which is an important risk factor of colorectal cancer, regardless of *H pylori* infection.

Peer review

The authors examined the relation between gastric dysplasia and *H pylori* and advanced colorectal adenoma. However, a large prospective case-controlled study is required to determine whether the presence of gastric dysplasia is an indication for colonoscopy and what intervals would be recommended for patients with gastric dysplasia.

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Total laparoscopic liver resection in 78 patients

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Abstract

AIM: To summarize the clinical experience of laparoscopic hepatectomy at a single center.

METHODS: Between November 2003 and March 2009, 78 patients with hepatocellular carcinoma ($n = 39$), metastatic liver carcinoma ($n = 10$), and benign liver neoplasms ($n = 29$) underwent laparoscopic hepatectomy in our unit. A retrospective analysis was done on the clinical outcomes of the 78 patients.

RESULTS: The lesions were located in segments I ($n = 3$), II ($n = 16$), III ($n = 24$), IV ($n = 11$), V ($n = 11$), VI ($n = 9$), and VIII ($n = 4$). The lesion sizes ranged from 0.8 to 15 cm. The number of lesions was three ($n = 4$), two ($n = 8$) and one ($n = 66$) in the study cohort. The surgical procedures included left hemi-hepatectomy ($n = 7$), left lateral lobectomy ($n = 14$), segmentectomy ($n = 11$), local resection ($n = 39$), and resection of metastatic liver lesions during laparoscopic surgery for rectal cancer ($n = 7$). Laparoscopic liver resection was successful in all patients, with no conversion to open procedures. Only four patients received blood transfusion (400-800 mL). There were no perioperative complications, such as bleeding and biliary leakage. The liver function of all patients recovered within 1 wk, and no liver failure occurred.

CONCLUSION: Laparoscopic hepatectomy is a safe and feasible operation with minimal surgical trauma. It should be performed by a surgeon with sufficient experience in open hepatic resection and who is proficient in laparoscopy.

INTRODUCTION

Laparoscopic hepatectomy (LH) was first described in 1995 by Cuesta *et al*^[1] and it remains an appealing concept: major surgery with a potential for bleeding, carried out using a minimally invasive approach. While the laparoscopic approach has been championed, notably by Cherqui *et al*^[2] and Buell *et al*^[3], it is carried out in only a few centers and has not become a widely accepted surgical technique. In general, laparoscopic liver resection offers some advantages over an open approach. There is less postoperative pain, early mobilization, minimal ileus, earlier resumption of oral intake and shorter hospital stay^[4-7]. Initial minor hepatectomy for lesions located in superficial segments has been considered possible^[8,9], with the development of laparoscopic instruments and parenchymal transection devices, combined with improved understanding of the vascular anatomy of the liver, it is now accepted widely that laparoscopy will be used increasingly in liver surgery^[10].

Between November 2003 and March 2009, we performed total laparoscopic hepatectomy in 78 patients with liver neoplasms, the results of which were encouraging.

MATERIALS AND METHODS

Patients

Between November 2003 and March 2009, 78 LHs were performed in the Hepato-biliary Surgery Department at Sun Yat-sen Memorial Hospital of Sun Yat-sen University. Seventy-eight patients with liver neoplasms

(50 men and 28 women), including primary liver carcinoma ($n = 39$), secondary liver carcinoma ($n = 10$), and benign liver neoplasm ($n = 29$). The mean age was 46.6 years (range, 20-82 years). Preoperative liver function was as follows: Child classification A ($n = 52$), B ($n = 22$) and C ($n = 4$). Evidence of cirrhosis, based on clinical and/or morphological abnormalities, was not considered an absolute contraindication in the absence of decompensated cirrhosis. There was no upper limit on neoplasm size, and LH was contraindicated if venous or biliary reconstruction was required.

Surgical procedure

All procedures were performed under general endotracheal anesthesia and after obtaining informed consent. All resections were performed with the patient in the supine position. The surgeon stood between the patient's spread legs, the first assistant on the left side of the patient and the camera assistant on the right. The pneumoperitoneum was insufflated by umbilicus puncture and controlled electronically at a constant abdominal pressure of 12 mmHg. A 5-mm trocar placed at the umbilical port was used for abdominal exploration with a 30° laparoscope. The trocar insertion sites depended on the location of the hepatic lesion. The surgical technique was as follows. For mobilization of the liver, the falciform and round ligaments were divided by an endoscopic ultrasonic scalpel (LLC, Guaynabo, Puerto Rico). In the case of left hepatectomy, the left liver lobe was mobilized by dividing the left triangular, coronary, and hepato-gastric ligaments. In the case of right segmentectomy, the right coronary ligament was divided. It was not necessary to divide the ligament if the lesion was located at the edge of the liver. Laparoscopic ultrasonography (Esaote Biomedica, Genoa, Italy) was used for localization of the tumor and the supply vessels, demonstration of satellite nodules, and demarcation of an adequate tumor-free margin. To achieve afferent blood control in anatomical left liver resection, including left segmentectomy, left lateral lobectomy, and left hemihepatectomy, the portal pedicles were dissected outside the liver parenchyma, and the portal venous branch, hepatic arterial branch, and bile duct were separated, clipped and divided. When the branch was too large to apply clips, it was divided with an endoscopic linear stapler (Tyco Healthcare, Norwalk, CT, USA). In right liver resection, the supply vessels of the target segment were found by laparoscopic ultrasonography, and were clipped or divided with an endoscopic linear stapler extraparenchymally. To achieve efferent blood control, the inferior vena cava and left hepatic vein were dissected, and the trunk of the left hepatic vein was clipped with a 12-mm hemoclip, but was not divided before parenchymal division. Liver surface tissue transection was performed using an endoscopic ultrasonic scalpel, while deep parenchyma dissection was performed using LigaSure (Valleylab, Boulder, CO, USA); larger biliary and vascular radicals were clipped and divided. After careful hemostasis, fibrin glue sealant (FibinGluRAAS; Shanghai Raas Blood Products, China) was applied to the raw surface. The specimen was extracted using a plastic bag through an extending port site.

Table 1 Tumor sites in 78 laparoscopic hepatectomy (LH) procedures

	<i>n</i>
Segment I	3
Segment II	16
Segment III	24
Segment IV	11
Segment V	11
Segment VI	9
Segment VII	4
Total	78

Assessment of operation

Surgical complications included biliary fistula, hemorrhage and incisional hernia. All patients underwent liver function tests and abdominal computed tomography (CT) before discharge. All patients underwent follow-up examination at 1-mo intervals after operation, and the follow-up examinations included clinical examination, liver function tests, and abdominal ultrasonography. No patient was lost at follow-up.

RESULTS

Operative results

Hepatic resection was performed laparoscopically in all 78 patients. There were no operative deaths and no conversions to laparotomy. The mean operation time was 165 min (range, 60-390 min). The mean blood loss was 288 mL (range, 10-1000 mL); only four patients received blood transfusion (400-800 mL). The mean size of the lesions was 6.2 cm (range, 0.8-15 cm) and the mean width of the specimen margins was 1.2 cm (range, 0.5-6 cm). The number of lesions was three ($n = 4$), two ($n = 8$), and one ($n = 66$) in the study cohort. Details of tumor sites are summarized in Table 1; Types of laparoscopic hepatectomy are given in Table 2; Details of histologic results are summarized in Table 3.

Postoperative recovery

There were no postoperative deaths or complications, such as bleeding and biliary leakage. Liver function returned to preoperative levels within 1 wk. Oral intake was begun on postoperative day 2. The mean postoperative hospital stay was 5.6 d (range, 2-10 d). The patients were discharged without narcotic analgesia. At the first postoperative month, all patients had resumed normal activities.

Follow-up

The mean follow-up was 30 mo (range, 2-60 mo). No port site metastasis was noted during follow-up in any patient who had hepatectomy for malignant lesions. Recurrence was detected in four patients with primary hepatocellular carcinoma and one with intrahepatic cholangiocarcinoma within 2 years. Two patients with primary hepatocellular carcinoma underwent a second LH for a recurrent lesion, and the other patients had transcatheter arterial chemoembolization and radiofrequency ablation (RFA). New isolated lesions in the liver were detected in two patients with rectal carcinoma within 1 year postoperatively, and were treated with RFA.

Table 2 Types of LH *n* (%)

	<i>n</i>
Anatomical resection	32 (41)
Left hemi-hepatectomy	7
Left lateral hepatic lobectomy	14
Hepatic segmentectomy	11
Local resection	39 (50)
Local liver resection for metastasis and colorectal laparoscopic resection	7 (9)
Total	78

Table 3 Histological results in 78 LH procedures *n* (%)

	<i>n</i>
Malignant tumor	52 (67)
Hepatocellular carcinoma	33
Intrahepatic cholangiocarcinoma	6
Colorectal metastasis	10
Gastric carcinoma metastasis	1
Ovarian carcinoma metastasis	1
Kidney carcinoma metastasis	1
Benign tumor	26 (33)
Haemangioma	21
Focal nodular hyperplasia	3
Granuloma	1
Adenoma	1
Total	78

DISCUSSION

Laparoscopic liver resection has been viewed with skepticism because of concerns regarding parenchymal transection, bleeding control, bile leakage and incomplete resection^[3,11,12]. The present study suggests that LH may be safe and feasible in selected patients. The indication for laparoscopic liver resection should be selected with care because of the technical difficulty, and the size and location of the neoplasm must be evaluated before surgery. In patients with neoplasms located in the left liver lobe and the anterior and inferior liver segments (IVb, V and VI), LH has been shown to be safe. On the other hand, neoplasms located in the right lobe and the posterior and superior liver segments (I, IVa, VII and VIII) are technically more demanding and should be approached with caution^[13,14]. In our cohort, most lesions (91%) were located in segment II, III, IV, V and VI, and no lesion located in segment VII was treated because of the difficulty of exposure. A neoplasm < 6 cm in size is thought to be preferred^[15,16]. In fact, we believe the size of the neoplasm is not as important as its location for LH. We performed laparoscopic left hemi-hepatectomy in four patients with 10-15-cm neoplasms in the left liver lobe, but without invasion of the inferior vena cava and the root of the hepatic vein. No conversion to laparotomy in our cohort may have been, at least in part, because of the choice of indication before surgery.

One of the main concerns during hepatectomy is minimizing blood loss and avoidance of blood transfusion^[17,18]. In our experience, laparoscopic surgery may provide better visualization of deep vascular structures and more precise and accurate surgery. To avoid injury



Figure 1 Computed tomography (CT) image before operation, which showed the lesion on the right lobe of the cirrhotic liver.



Figure 2 After laparoscopic hepatectomy (LH), the lesion disappeared and the volume of liver increased clearly.

to the hepatic veins, caution should be exercised during manipulation of secondary hilar structures, and the hepatic veins should be transected in the parenchyma using clips or an endoscopic linear stapler. Conversion to laparotomy is mandatory when a large venous injury occurs, even if initial laparoscopic control of the wound has been achieved^[11]. In the early stage of LH, bleeding during parenchymal transection due to a lack of effective devices is another important cause of blood loss, but there are now numerous excellent devices for dividing the parenchyma, including an ultrasonic scalpel, microwave tissue coagulator, water jet dissector, LigaSure, Cavitron ultrasonic surgical aspirator, argon beam coagulator, Peng's Multifunction Operative Dissector, and TissueLink^[19-26]. Based on our experience, using an ultrasonic scalpel and LigaSure to transect the liver facilitates good hemostasis, clear anatomy, less effusion, and minor damage to liver function. The average blood loss was only 200 mL in our 78 cases. Another concern that occurs during LH when resecting a malignant neoplasm involves achieving a disease-free resection margin^[27,28]. We believe a 1-cm free surgical margin can be obtained using laparoscopic ultrasonography. In our research and a European multicenter study, there was no evidence that the use of a laparoscopic technique increases the risk of local recurrence and port-site metastases^[29,30]. LH carries the potential risk of gas embolism caused by a pneumoperitoneum during hepatic vein division^[31]. Although this potential complication

appears to be rare, we utilized several precautions to avoid embolic events, including careful dissection of the hepatic vein, a low-pressure pneumoperitoneum, and preventive obstruction of the left hepatic vein with a 12-mm hemoclip.

LH has the advantage of minimal scarring and fewer adhesions, thereby increasing the feasibility of repeat liver resection. We performed repeat laparoscopic liver resections in two patients for recurrent lesions, and the results were satisfactory. LH may very well become the gold standard for tumor control in patients with hepatocellular carcinoma awaiting liver transplantation^[32]. The choice of an incision is a dilemma in the colorectal cancer patient complicated by liver metastasis. The present study has shown that simultaneous laparoscopic resection of colorectal cancer and synchronous liver metastases is feasible and safe. Indeed, we performed this operation successfully in seven patients with five or six ports, just one or two more than laparoscopic resection of colorectal cancer, and no complications occurred. LH also has an irreplaceable advantage in patients with poor liver function. We performed LH in four patients with cirrhosis and Child C liver function in whom liver resection *via* laparotomy carried a high risk of complications. They all recovered rapidly without development of ascites and jaundice. In one hepatocellular carcinoma patient with cirrhosis and hepatitis C infection, liver function did not improve following medical treatment, yet returned to normal within 5 d after local laparoscopic tumor resection. LH might improve the postoperative course of patients with cirrhosis for the following reasons: (1) preservation of the abdominal wall and the round ligament avoids interruption of collateral circulation; (2) less mobilization and manipulation of the liver reduces liver trauma; and (3) non-exposure of abdominal viscera restricts fluid requirements and decreases electrolytic and protein losses^[33-35]. We suggest that local laparoscopic liver tumor resection in patients with cirrhosis not only treats the tumor, but also creates a damaged liver environment with minimal liver load, which provides ideal conditions for regeneration of remnant liver. In fact, with CT scanning, we confirmed that the volume of the liver increased after LH in these patients (Figures 1 and 2).

Lastly, we advocate LH as a complex procedure that requires experience and different skills from those of open surgery because of the 2D representation of the operative site, limited tactile feedback, and the need for eye-hand coordination skills. The surgeons in our study all had sufficient experience with open hepatic resection and proficient skills in laparoscopic procedures, and also had the ability to manage the various complications of liver resection.

COMMENTS

Background

With the development of laparoscopic instruments and parenchymal transection devices, combined with the improved understanding of the vascular anatomy of the liver, it is now accepted widely that laparoscopy will be used increasingly in liver surgery.

Research frontiers

The underlying intent of laparoscopic surgery for liver neoplasms is to provide curative resection while minimizing complications. In this study, the authors

demonstrated that laparoscopic hepatectomy (LH) could be safe and feasible, with minimal trauma.

Innovations and breakthroughs

This manuscript described the retrospective evaluation of a significant cohort of patients who underwent LH at a single center. The good clinical outcome depended on the reasonable choice of indication, suitable parenchymal transection devices, and sufficient experience in hepatic resection and laparoscopy.

Applications

LH could be performed in most patients with liver neoplasms, particularly in patients with cirrhosis and those with colorectal cancer and synchronous liver metastases.

Terminology

Liver resection is always considered a major operation, with blood loss and lengthy hospitalization. LH could benefit patients in need of liver resection, with better cosmetic results, less postoperative pain and shorter hospital stay.

Peer review

Interesting report about a single centre experience with 78 laparoscopic liver resections of different kind for different indications in different liver diseases.

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BRIEF ARTICLE

A simple taurocholate-induced model of severe acute pancreatitis in rats

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Author contributions: Peng JS conceived and designed the study; Liu ZH, Li CJ, Song H, Wu XB, Chen JR and Diao DC carried out the experiment; Liu ZH drafted the manuscript; Yang ZL and Xiang J performed data analysis and revision of the manuscript.

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appeared to be destroyed with loose, shortened microvilli and rupture of the intercellular junction, as shown by electron microscopy.

CONCLUSION: Significant gut barrier damage and intestinal bacterial translocation were definitely observed with few potential study confounders in this SAP rat model, suggesting that it may be an appropriate animal model for study of gut barrier damage and bacterial translocation in SAP.

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Key words: Acute pancreatitis; Bacterial translocation; Inflammation; Real-time polymerase chain reaction

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Abstract

AIM: To investigate gut barrier damage and intestinal bacteria translocation in severe acute pancreatitis (SAP), a simple rat model of SAP was induced and studied.

METHODS: Pancreatitis was induced by uniformly distributed injection of 3.8% Na taurocholate (1 mL/kg) beneath the pancreatic capsule. Rats in the control group were injected with normal saline in the identical location.

RESULTS: Serum amylase, plasma endotoxin, intestinal permeability, and pancreatitis pathology scores were all markedly higher in the pancreatitis group than in the control group ($P < 0.01$). The bacterial infection rate was significantly higher in the SAP group than in the control group ($P < 0.01$), observed in parallel by both bacterial culture and real-time polymerase chain reaction. Acute damage of the pancreas was observed histologically in SAP rats, showing interstitial edema, leukocyte infiltration, acinar cell necrosis and hemorrhage. The microstructure of the intestinal mucosa of SAP rats

INTRODUCTION

A close link has been demonstrated between intestinal bacteria translocation and infection in pancreatic necrosis, which is a main predictor of clinical outcome in patients with severe acute pancreatitis (SAP)^[1]. Although a multitude of animal models^[2-5] have been used to study the mechanism of bacterial translocation, its exact origin, route, and mechanism are still unclear. The main reason for this uncertainty is the lack of an "ideal" animal model of acute pancreatitis (AP), especially for the purpose of studying bacterial translocation.

Taurocholate-induced pancreatitis animal models are commonly used nowadays, especially the model induced by perfusion of taurocholate into the biliary or pancreatic duct, which is thought to most closely resemble clinical biliary pancreatitis. However these models have some shortcomings such as prolonged

preparation time, difficult operation, high death rate, and potential infection of the pancreas when puncturing through the gut, which limit their further popularity in study of bacterial translocation in SAP.

To overcome these shortcomings, in this paper we induced a taurocholate pancreatitis rat model by uniformly distributed injection of taurocholate directly beneath the pancreatic capsule. Gut barrier damage and intestinal bacterial translocation were studied and the advantages of the model were characterized.

MATERIALS AND METHODS

Animals and animal procedures

Thirty two specific pathogen-free rats (half male and half female, obtained from the Experimental Animal Center of Guangdong Province, China), were randomly divided into 2 groups (16 rats in the experimental group, 16 in the control group). Average weight at the time of the experiment was 250 ± 20 g. Anesthesia was performed with intraperitoneal injection of 10% chloral hydrate 3 mL/kg.

The study and all procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, China.

Model induction procedure

Pancreatitis group: rats were fasted for 12 h before the operation. A 10 mm median laparotomy was made and the pancreas was well exposed. The pancreatic capsule was gently lifted up, and 3.8% Na taurocholate 1 mL/kg was injected beneath the pancreatic capsule and distributed by making a bubble of taurocholate, about 2-3 mm in diameter at every injection point. Several minutes later, the pancreatic tissue would become dark purple, swell, and bleed locally. Then the abdominal organs were gently repositioned, and the peritoneal cavity was doused with about 50 mL of normal saline. Finally the abdominal wall was sutured.

In the control group, rats were injected similarly with normal saline 1 mL/kg in place of Na taurocholate.

Post-surgery breeding

After surgery, rats were housed in a dry and clean room at a temperature of 20-26°C, and 12 h later standard chow and water were freely available. Mortality was observed and recorded every day.

Determination of serum amylase and plasma endotoxin level

Serum amylase and plasma endotoxin were detected by routine clinical chemistry methods. Serum amylase was measured at 3 time points, namely 6, 24 and 48 h. On the 4th and 7th days, plasma endotoxins of every live rat were measured.

Determination of gut permeability by measurement of serum fluorescent isothiocyanate dextran (FITC-Dextran) in the superior mesenteric vein (SMV)

FITC-Dextran (Sigma), a polysaccharide macromolecule with a fluorescent marker, fluorescein isothiocyanate, can-

not be digested and absorbed when in the normal alimentary tract, but can pass through the damaged gut barrier into capillaries of the gut, and finally into the blood of the SMV when intestinal permeability is increased^[6-9]. The concentration of FITC-Dextran in the SMV depends on the dose of the drug and the severity of gut barrier damage. In this study, rats were perfused with 1 mL of 0.5% FITC-Dextran through a small tube placed in the stomach *via* the mouth, and 12 h later SMV blood samples were taken to measure the FITC-Dextran concentration as follows: blood was centrifuged at 1000 r/min, the supernatant was obtained, and absorbance was detected on a spectrofluorometer (Thermo Ltd., USA) at an excitation wave of 490 nm and transmission wave of 520 nm; the concentration of FITC-Dextran was finally calculated according to the standard curve of FITC-Dextran.

Bacterial culture for detection of infection rates and real-time polymerase chain reaction (PCR) to quantify the total number of translocated bacteria

One millilitre of blood from the inferior vena cava, 0.1 g mesenteric lymph node (MLN) tissue, 0.3 g pancreas tissue and 1 g liver tissue were taken from each rat under sterile conditions by laparotomy. Every sample had 1 mL saline added followed by homogenization by triturating, then uniform smearing on 4 brain heart solid culture medium (Hopebiotechnology Co. Ltd., Qingdao, China), 2 of which were placed in a 37°C thermostat, the other 2 were placed in an anaerobic jar, tightly closed and put in a 37°C thermostat. Results were observed and recorded 48 h later.

The same quantities of samples as above were taken for real-time PCR. Total bacterial genomic DNA was extracted by the DNeasy Blood & Tissue kit (Cat. 69506, Qiagen, USA) according to the manufacturer's instructions. Li *et al.*^[10] confirmed the appropriateness and effectiveness of this kit for studying gut microbial ecology. The universal PCR primer sets for total bacteria (forward: TCCTACGGGAGGCAGCAGT; reverse: GGACTACCAGGGTATCTAATCCTGTT) used in this study were designed for a region in 16S rRNA and had been well demonstrated by other researchers to be uniformly successful in detecting a wide range of bacteria in samples of dentine^[11] and feces^[12]. Real-time PCR was performed with the ABI-Prism 7900 Sequence Detection System (Applied Biosystems, USA). The PCR reaction was performed in a total volume of 25 μ L, containing SYBR Green Real-time PCR Master Mix (TOYOBO Inc., Japan) 12.5 μ L, with 200 nmol/L of each of the forward and reverse primers and 2 ng of DNA for each reaction. The PCR reaction conditions for amplification of DNA were 95°C for 1 min and 40 cycles of 95°C for 20 s and 60°C for 1 min. Data analysis was performed using Sequence Detection Software supplied by Applied Biosystems.

Pancreatic pathology

The pancreas was cut, formalin-fixed and embedded in paraffin, and 4 μ m sections were cut and stained with hematoxylin and eosin. The pancreatitis pathology score of every rat at both the 4th and 7th days were

Table 1 Pancreatitis histopathology: Schmidt Scoring Criteria

	Score			
	0	1	2	3
Interstitial edema	None	Interlobular	Lobule involved	Isolated island-like acinar cells
Leukocyte infiltration	None	< 20%	20%-50%	> 50%
Acinar cell necrosis	None	< 5%	5%-20%	> 20%
Hemorrhage	None	1-2 points	3-5 points	> 20%

The Schmidt Scoring Criteria of pancreatitis pathology includes 4 aspects, including interstitial edema, inflammatory infiltration, parenchymal necrosis and hemorrhage, all of which were given scores of 0-3 according to the severity of the disease.

determined according to the severity of pancreatic damage by a pathologist using a double-blind method, according to the Schmidt Scoring Criteria^[13] (Table 1).

Intestinal mucosa electron microscopy

The 4th day, which was the mid-term course of the illness, was chosen to determine the microstructural changes in the intestinal mucosa. In order to sample from the same intestinal location of all rats, a 2 cm section of the distal ileum (next to the ileocecal valve) was taken and quickly placed in fixative solution, and sent to the Electron Microscopy Center of Sun Yat-sen University for electron microscopy examination.

Animal mortality

Daily observation of animal deaths was made for 7 d. The cumulative mortality rate was calculated daily.

Statistical analysis

Data were displayed as the mean $\pm$ SE. Statistical significance was calculated by one-way analysis of variance or the χ^2 test, using the Sigma Plot Software Package (Systat Software Inc, Point Richmond, Ca, USA).

RESULTS

Abnormal performance of rats

Pancreatitis group: Several minutes after injection of taurocholate beneath the pancreatic capsule, the pancreas was found to become swollen, dark purple, with partial subcapsular hemorrhage. The rats took more time to wake after surgery than control rats, and were tired on the first day with reduced activity, no appetite, and slow responses. Conditions became worst on the 2nd and 3rd days, when some rats were lethargic, had conjunctival congestion or petechiae, abdominal distention and hyperventilation and some rats died. However after the 4th day, the surviving rats would always recover gradually.

Control group: Rats awoke soon after operation, recovered in 6-8 h with normal characteristics and activity. 24 h later the rats performed as normal, and with no deaths.

Feasibility of the surgical procedure

Uniformly distributed injections beneath the pancreatic

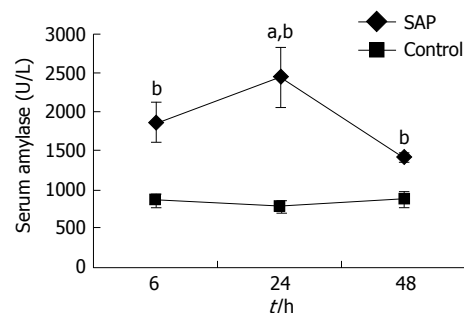


Figure 1 Amylase level in serum. Compared with control animals, serum amylase levels in severe acute pancreatitis (SAP) rats were higher at each time point (6, 24 and 48 h), ^a $P < 0.01$. At 24 h, the amylase level of the pancreatitis model reached a peak, and was higher than the other 2 time points, ^b $P < 0.05$.

capsule were successfully achieved in all animals and required an average of 15 ± 2 min per animal when the technique was well mastered. The cumulative death rate at 7 d in the pancreatitis group was about 30%, with most occurring on the 3rd and 4th days (about 80% of all), while no deaths occurred in the control group.

There were 2 main points of note during the operation. First, a puncture too deep into the pancreatic parenchyma could injure the pancreatic veins and cause direct bleeding, but simply pressing with aseptic gauze for a moment would stop this. Second, if the syringe needle pierced through the pancreatic capsule, or taurocholate bubbles under the pancreatic capsule burst, the taurocholate could go into the abdominal cavity and cause biliary peritonitis. However, as a whole, the operation itself was simple and safe, causing few complications.

Rat autopsy

Autopsies were carried out on dead rats. In all cases, much yellow or bloody exudate with a slight smell would come out of the peritoneal cavity when the abdomen was opened. The intestine would expand to 2-3 times of normal with gas and fluid inside and with a thinner wall. The pancreas was swollen and dark colored with some local hemorrhagic spots. Yellow-white saponified spots were always found in the peripancreatic omentum and retroperitoneum. The lower part of the spleen was always dark black. A pancreatic or hepatic abscess could be observed if the rat died after the 4th day or later.

Serum level of amylase

In pancreatitis rats, compared with the control group, serum amylase began increasing 6 h after the operation, and the level reached a peak at 24 h, remaining higher than the control group 48 h later (Figure 1).

Pancreatic histology

Microscopic pancreatic damage was pronounced in pancreatitis animals in the first 48 h and showed a progressive disease course; 6 h after pancreatitis induction, typical pathological features were edema and hemorrhage (Figure 2B), while apparent leukocyte infiltration was observed 24 h after operation (Figure 2C), and areas of acinar cell necrosis could be observed 48 h after operation

Table 2 Pancreatitis pathology score

Groups (<i>n</i> = 8)	Score				
	Interstitial edema	Leukocyte infiltration	Acinar cell necrosis	Hemorrhage	Total
Control					
4 d	0.13 ± 0.33	0.13 ± 0.33	0	0	0.25 ± 0.67
7 d	0.13 ± 0.33	0	0	0	0.13 ± 0.33
SAP					
4 d	1.5 ± 0.76 ^a	2.38 ± 0.74 ^a	1.75 ± 0.71 ^a	0.75 ± 1.17 ^a	6.38 ± 2.20 ^a
7 d	1.25 ± 0.46 ^b	2.13 ± 0.99 ^b	1.5 ± 0.54 ^b	0.50 ± 1.07 ^b	5.38 ± 1.77 ^b

In this table, the pancreatitis pathology scores in severe acute pancreatitis (SAP) rats were significantly higher than in control rats in every single aspect as well as the total score, both on the 4th day (^a*P* < 0.01) and on the 7th day (^b*P* < 0.01).

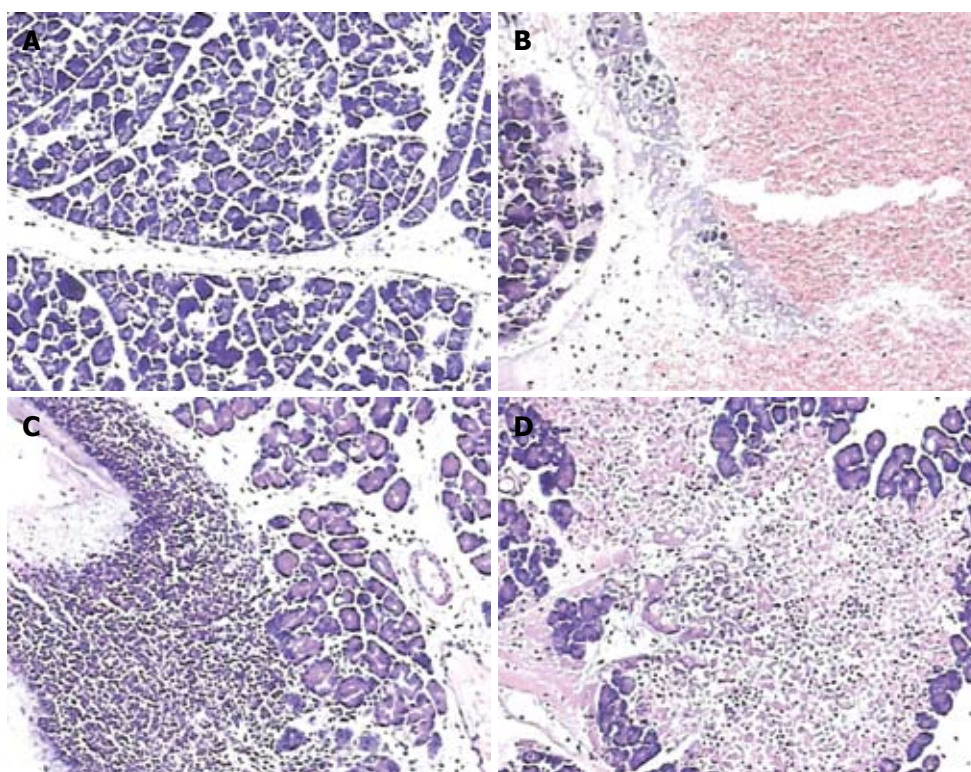


Figure 2 Pancreatic pathology (HE, × 100). A: Normal pancreatic histology; B: Pancreatic damage induced 6 h after pancreatitis induction. Typical pathological features were edema and hemorrhage; C: Apparent leukocyte infiltration was observed 24 h after surgery; D: Pancreatic pathology induced 48 h after surgery, showing a confluent area of acinar cell necrosis.

(Figure 2D). The control animals were observed without such abnormalities, and had normal pancreatic gland lobules and cells. (Figure 2A)

On both the 4th and 7th days, pancreatitis pathology scores according to the Schmidt criteria were observed to be significantly higher in the pancreatitis group than in the control group in all 4 single aspects, as well as the total score (*P* < 0.01, Table 2), showing that significant damage had occurred in the pancreas of SAP rats.

Serum endotoxin

The serum endotoxin level was a sign of intestinal permeability and pancreatitis severity. The plasma endotoxin levels in the pancreatitis group were about 5-7 times higher than that in the control group on both the 4th and 7th days (*P* < 0.01), though the level had decreased almost a half on the 7th day compared to the 4th day (Figure 3).

Intestinal permeability

FITC-Dextran concentration in the SMV was a sign of intestinal permeability. In this study, FITC-Dextran levels in the SMV of pancreatitis rats were observed to be significantly higher than that of control rats on both the 4th and 7th days (*P* < 0.01, Figure 4), indicating higher intestinal permeability in the pancreatitis rats.

Ultrastructural changes of the iliac mucosa

Microvilli of iliac mucosa in pancreatitis rats (Figure 5A) were shortened, about half the length of those of the control rats (Figure 5C), and with a loose structure, and some points had disappeared. Tight junctions and intermediate junctions of villous cells in SAP rats were unclear with discontinuities (Figure 5B), while intact and clear junctions between villous cells were seen in control rats (Figure 5D).

Table 3 Infection determined by the bacterial culture method

Groups (n = 8)	Infected organs number														
	Blood			MLN			Pancreas			Liver			Total		
	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T
Control															
4 d	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1
7 d	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SAP															
4 d	3	2	3	6	6	6	7	7	7	7	7	7	23	22	23 ^a
7 d	3	3	3	5	6	6	7	8	8	5	5	5	20	22	22 ^b
Total	6	5	6 ^c	11	13	13	14	15	15	12	12	12	43	45	46

Infection rates of each organ and overall in the SAP group were higher than that in the control group, both on the 4th day (^a $P < 0.01$) and 7th day (^b $P < 0.01$); Compared with the other 3 types of organ, the infection rate of blood was lowest, ^c $P < 0.05$. MLN: Mesenteric lymph nodes; Ae: Aerobes; Ana: Anaerobes; T: Total.

Table 4 Bacterial translocation detected by real-time polymerase chain reaction

	Samples (n = 16)			
	Blood	MLN	Pancreas	Liver
Infected number in control group	0	1	0	0
Infected number in SAP group	5 ^a	10 ^a	13 ^a	12 ^a
Bacteria number (transformed by log ₁₀)	5.43 ± 0.847	6.48 ± 1.56	7.70 ± 0.766 ^b	6.57 ± 1.44

^a $P < 0.01$, compared with the "Infected number" in the control group; ^b $P < 0.05$, compared with the "Bacteria number (transformed by log₁₀)" in blood, MLN and liver.

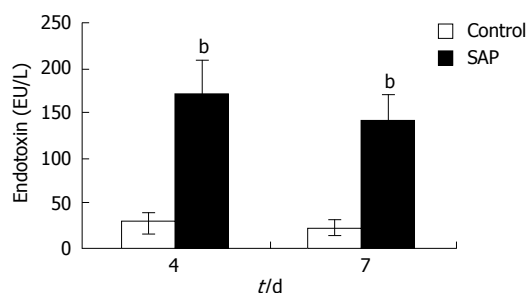


Figure 3 Endotoxin level in serum. On the 4th and 7th days, the serum endotoxin level was markedly higher in the pancreatitis group compared to the control group, ^b $P < 0.01$.

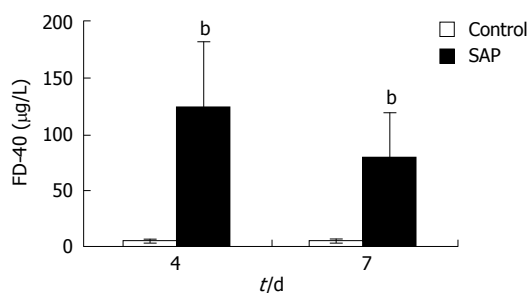


Figure 4 FITC-Dextran (FD-40) levels of serum in the superior mesenteric vein (SMV). The serum level of FD-40 in SMV was significantly higher in the pancreatitis group than in the control group on the 4th and 7th days, ^b $P < 0.01$, though the level had decreased almost a half between days 4 and 7.

Bacterial culture results

The copies of translocated bacteria differed greatly among pancreatitis rats, sometimes by 10^3 times, so it was difficult to define the statistics on bacterial numbers by the bacterial culture method. Therefore we chose the infection rate as a variable to compare the bacterial translocation in the 2 groups by the agar plate culture method. Results showed that infection rates of all the 4 types of organ were higher in the pancreatitis group than in the control group ($P < 0.01$). When comparison was made among the infected rats of the 4 kinds of organs, blood of the inferior vena cava was found to have the lowest rate ($P < 0.05$, Table 3).

Real-time PCR results of bacterial translocation

The infection rates detected by real-time PCR were mainly the same as that by bacterial culture. Infection rates by the culture method in MLN and pancreas were a little higher than that by real-time PCR method (MLN 12 to 10,

pancreas 15 to 13), which may result from contamination during culture. An advantage of the real-time PCR method was its accuracy of bacterial enumeration. In this study, the results of bacterial number had been transformed by log₁₀ for the purpose of better statistics. It was observed that the pancreas had the highest number of bacteria of the 4 kinds of organs ($P < 0.05$), which may relate to necrosis and hemorrhage in pancreas tissue as a good medium for bacteria breeding (Table 4).

DISCUSSION

Pancreatitis is a distressing disease clinically, especially SAP, which is associated with considerable mortality, as high as 30%-40%. The main features of this condition are pancreatic necrosis and associated sepsis, with both localized and systemic inflammatory response syndromes^[1,14]. Infection of pancreatic necrosis is regarded to be

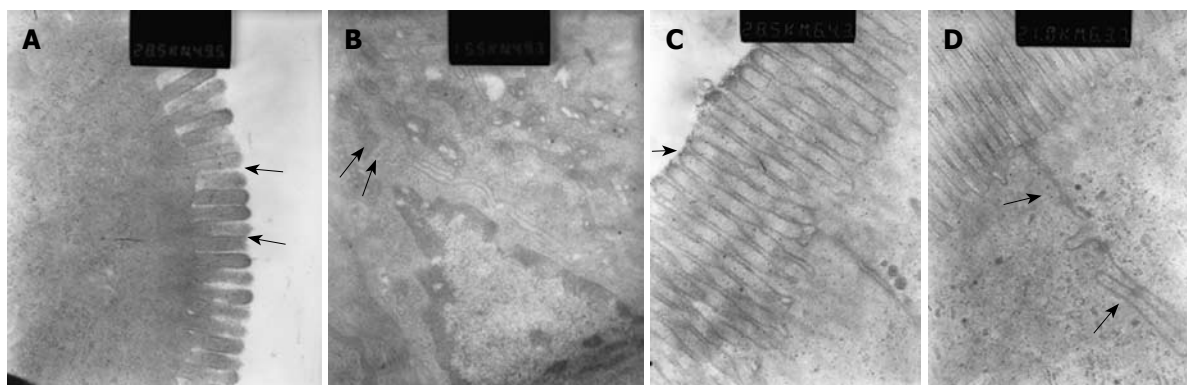


Figure 5 Distal iliac mucosa electron microscopy. A: Changes of microvilli in a SAP rat, showing loose, shortened microvilli, with some points lost (arrows) (Original magnification, $\times 28.5$ K); B: Changes of the intermediate junction in a SAP rat, which was unclear with breakage in some parts (arrows) ($\times 15.5$ K); C ($\times 28.5$ K) and D ($\times 21.0$ K) are representative electron microscopy images of the iliac mucosa in control rats, showing long, tight microvilli (arrow) and intact intermediate junctions (arrows).

a main predictor of outcome during SAP, and bacterial translocation of intestinal flora is considered to be the cause of pancreatic infection^[15].

Since Bernard first introduced an AP model of rabbits by injection of bile and olive oil into the pancreatic duct 150 years ago, animal experiments have become an irreplaceable method to study the pathophysiology, diagnosis and treatment of AP which, compared with clinical trials, have many advantages such as accessible subjects, standardization of the degree of the lesion, practicability of invasive inspection, adequate tissue samples, practicability of prophylactic treatment.

Nowadays there are many kinds of experimental models used to study bacterial translocation in AP. An ideal model of AP should encompass several features including easy reproducibility, with the ability to vary the severity of AP in a standardized manner according to the experimental aim, and to mimic the morphology and pathophysiology of the human situation^[16]. At present, several commonly used experimental models were developed to imitate the human biliary pancreatitis, including duodenal loop, biliopancreatic duct ligation, and biliopancreatic duct perfusion.

A duodenal loop in rats can lead to AP of varying severity^[17]. However, block of the gastrointestinal tract leads to mucosal atrophy and functional changes to the mucosal barrier^[18]. Obstruction of bile flow into the intestine was shown to reduce intestinal motility, causing small bowel bacterial overgrowth and increased bacterial translocation^[19-21]. Furthermore, the reflux of duodenal contents, including bacteria, into the biliopancreatic duct will confound the bacterial infection of the pancreas. All these limit its popularity.

The duct ligation model, in which the common biliopancreatic duct is surgically clipped or tied at the sphincter of Oddi complex, could produce moderate pancreatitis with minimal acinar cell necrosis only in American opossum inducing SAP. There are several factors that limit its use. First, obstruction of bile flow into the intestine causes small bowel bacterial overgrowth and bacterial translocation^[20]. Second, induction of jaundice would impair the immune system to an uncertain degree and make the research result inaccurate. Third,

exclusion of pancreatic proteases in the gut lumen alters intestinal permeability, which does not truly occur in human pancreatitis^[22,23].

The duct perfusion model, usually made by perfusion of taurocholate after puncturing the duodenum and cannulating the papilla of Vater, was the most popular model of AP, with etiology resembling human biliary pancreatitis and with the advantage of quick induction of AP and reproducibility of results. However several potential confounders exist in it for study of bacterial translocation. The introduction of duodenal bacteria, through the papilla of Vater into the biliopancreatic duct could potentially affect the result of pancreatic infection; duodenal puncture and intestinal handling during surgery may also potentially affect the mucosal barrier function^[24]. A complicated surgical procedure and prolonged time are other shortcomings of it.

In 2002 Wu *et al.*^[20] first reported that pancreatic subcapsular injection of 3.8% Na taurocholate could establish an SAP animal model with multiple organ dysfunction in rats, but with a high mortality rate (90% in a week), which may be related to excessive use of 3.8% Na taurocholate (1 mL/rat) and of small rats (body weight about 100 g). This study used pancreatic subcapsular injection of 3.8% Na taurocholate (1 mL/kg) in rats of about 250 g body weight, and successfully established a model of AP. AP rats showed abnormal performance resembling human disease and the autopsy was in line with the performance of human SAP as well. The serum amylase level and pancreatic tissue pathology gave a clear diagnosis of AP. Changes of serum amylase levels in the AP group were similar to that in human disease, and increased significantly 6 h after induction, reaching a peak in 24 h and remaining at a higher level 48 h later. Pancreatitis pathology showed interstitial edema of the pancreas 6 h after model induction, with local hemorrhaging and slight inflammatory infiltration. Lesions developed progressively to moderate necrosis with obvious inflammatory infiltration 48 h later. The pancreatic pathology score on the 4th and 7th days showed a clear phenomenon of necrotic pancreatitis, including interstitial edema, leukocyte infiltration, acinar cell necrosis and hemorrhage. The scores of edema and hemorrhage in

the pancreatitis group were less than that of necrosis and inflammatory infiltration, which may be related to edema absorption and hematoma organization after several days. Some other organs were involved, peripancreatic calcified plaques formed, with liver and spleen ischemia, intestinal expansion, peritoneal exudates, and even death. Severity reached a peak in the medium term of the disease (3rd and 4th days), with the highest mortality, and although some indices were still high on the 7th day, few deaths occurred after 7 d, which may be related to the adaptation of the body or a sign of recovery. The cumulative mortality rate in 7 d was moderate, about 30%.

Agar plate culture is nowadays the gold standard method for bacterial measurement, however it has some shortcomings such as prolonged time and inaccuracy that limit early detection of bacterial translocation in the clinic. Recently, a real-time PCR method has been demonstrated successfully in the fast and accurate detection of bacteria in samples of blood, feces, and dentine; however few results are available on its use to detect bacteria in parenchymal organs such as lymphoid tissue, spleen, *etc.* Our study confirmed its use in enumeration of bacteria in this AP model in samples of not only blood but also parenchymal organs. Infection rates of all the 4 types of organ in the SAP group were significantly higher than that of the control group with quite similar results detected by bacterial culture or real-time PCR. Infection rate and bacterial number of inferior vena cava blood in the SAP group were both much lower than that of the other organs, while the MLN had a similar infection rate and bacterial number to that of the liver, suggesting a lymphatic route of bacterial translocation may exist in this SAP model.

A damaged intestinal barrier is thought to be a key factor for bacterial translocation in SAP^[25-28]. In this study, obvious gut barrier damage was observed, with the FITC-Dextran in the SMV and endotoxin levels both significantly increased in AP rats compared to control rats. Electron microscopy of the intestinal mucosa directly showed the damage in the mucosa cells, with microvillus atrophy and intercellular junction disruption.

This rat model requires an invasive laparotomy, with some inevitable potential confounding factors, such as anesthesia, trauma, stress and so on, but few other potential confounding factors existed in it. The surgical procedures were so simple that they could be finished in 15 min once the skills were mastered. No other organs except the pancreas were affected during the surgery, therefore avoiding some potential factors that could affect the measurement of bacterial translocation.

In conclusion, in this study we induced a simple taurocholate pancreatitis model in rats to study the situation of intestinal barrier damage and bacterial translocation in SAP. The results confirmed the situation, as observed clinically, that intestinal barrier damage and bacterial translocation exist in SAP with endotoxemia. However the exact pathophysiologic mechanism of bacterial translocation in SAP is still unknown and needs more intense research for the purpose of determining better preventative measures and treatment of SAP in the clinic.

COMMENTS

Background

Severe acute pancreatitis is associated with considerable mortality, as high as 30%-40%. Infection of pancreatic necrosis is regarded to be a main predictor of outcome during severe acute pancreatitis (SAP), and bacterial translocation of intestinal flora is considered to be the cause. Although a multitude of animal models have been used to study the mechanism of bacterial translocation, the exact origin, route, and mechanism of bacterial translocation causing infection of the necrotic pancreas are still unclear.

Research frontiers

There are now many kinds of experimental models used to study bacterial translocation in acute pancreatitis (AP). However, an "ideal" animal model of SAP was lacking, and should encompass several features including easy reproducibility, ability to vary the severity of AP in a standardized manner, and similar morphology and pathophysiology to that of the human situation.

Innovations and breakthroughs

A new simple model of SAP in the rat was induced by Na taurocholate, and results showed that significant gut barrier damage and intestinal bacterial translocation occurred in this SAP rat model, with few potential study confounders and with a shorter induction time compared to other commonly used animal models of SAP, suggesting that it may be an appropriate animal model for the study of gut barrier damage and bacterial translocation in SAP.

Applications

In future, this simple taurocholate-induced rat model may be used in the study of SAP, especially to determine the exact origin, route, and mechanism of bacterial translocation causing infection of the necrotic pancreas.

Terminology

Intestinal bacterial translocation: normally, intestinal bacteria cannot go through the gut barrier and into the blood or other organs. In the situation of SAP or other severe disease such as shock or major burns, the gut barrier would be damaged and the intestinal permeability increased, causing the intestinal bacteria to translocate from the gut to blood or even other distant organs and induce sepsis, septicemia or even death.

Peer review

This is a well performed research trying to induce a new simple rat model of SAP, and results definitely showed it was an appropriate animal model for study of bacterial translocation in SAP, with significant gut barrier damage and intestinal bacterial translocation observed but with few potential confounders.

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BRIEF ARTICLE

Single-center experience of 309 consecutive patients with obscure gastrointestinal bleeding

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Abstract

AIM: To investigate the diagnostic yield of capsule endoscopy (CE) in patients with obscure gastrointestinal bleeding (OGIB), and to determine whether the yield was affected by different bleeding status.

METHODS: Three hundred and nine consecutive patients (all with recent negative gastric and colonic endoscopy results) were investigated with CE; 49 cases with massive bleeding and 260 cases with chronic recurrent overt bleeding. Data regarding OGIB were obtained by retrospective chart review and review of an internal database of CE findings.

RESULTS: Visualization of the entire small intestine was achieved in 81.88% (253/309) of cases. Clinically positive findings occurred in 53.72% (166/309) of cases. The positivity of the massive bleeding group was slightly higher than that of the chronic recurrent overt bleeding group but there was no significant difference (59.18% vs 52.69%, $P > 0.05$) between the two groups. Small intestinal tumors were the most common finding in the entire cohort, these accounted for 30% of clinically significant lesions. In the chronic recurrent overt bleeding group angioectasia incidence reached more than 29%, while in the massive bleeding

group, small intestinal tumors were the most common finding at an incidence of over 51%. Increasing patient age was associated with positive diagnostic yield of CE and the findings of OGIB were different according to age range. Four cases were compromised due to the capsule remaining in the stomach during the entire test, and another patient underwent emergency surgery for massive bleeding. Therefore, the complication rate was 1.3%.

CONCLUSION: In this study CE was proven to be a safe, comfortable, and effective procedure, with a high rate of accuracy for diagnosing OGIB.

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Key words: Obscure gastrointestinal bleeding; Capsule endoscopy; Massive bleeding; Chronic recurrent overt occult bleeding

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as recurrent or persistent bleeding or iron deficiency anemia after a negative result from gastric and colonic endoscopy^[1]. OGIB accounts for approximately 5% of all gastrointestinal (GI) bleeding and lesions in the small bowel^[2]. Previous capsule endoscopy (CE) investigations of small intestine showed the value in finding small intestinal diseases, especially inflammatory bowel disease and OGIB. CE is a tool that allows for visualization of mucosa throughout the entire small bowel and it has already gained widespread clinical acceptance. CE has been shown to be superior to push enteroscopy^[3,4], small bowel follow-through^[5] and computed tomography

(CT) scan^[6] in detecting bleeding lesions in the small intestine. Early reports indicated that 45%-66% of patients with OGIB were detected by CE^[3,5,7]. Despite promising preliminary results, published studies were limited to small groups of patients. In this study a large group of OGIB patients were investigated by CE examination, including obscure-overt and a few obscure-occult patients. We divided these patients into a massive bleeding group (bloody stool or circulatory failure, serious anemia) and a chronic recurrent overt occult bleeding group (melena). Therefore, we investigated the outcome of CE in a large group of patients with OGIB from a single institution and determined whether the results of CE for OGIB patients were related to bleeding status. Also, the selection of OGIB candidates for successful CE evaluation was discussed in our study.

MATERIALS AND METHODS

Patients

Three hundred and nine cases underwent CE for the indication of OGIB at our institution from May, 2003 to April, 2008. All patients suffering at least two episodes of bleeding prior to CE had undergone endoscopy without localization of a bleeding lesion. Data of the 309 cases were obtained by retrospective chart review. Data included: age, gender, inpatient or outpatient status, status of GI bleeding (massive bleeding *vs* chronic recurrent overt occult bleeding), median units of red blood cells (RBCs) transfused, mean hemoglobin value prior to endoscopic evaluation, prior radiographic evaluation, computed tomography (CT) evaluation, and lesion diagnosis confirmed by surgery. CE data collected included: whether the entire small intestine was visualized, CE findings in the small intestine and whether complications occurred. Pregnant women, patients with pace-makers or intestinal obstruction were excluded from the study. The study was approved by the local ethics committees.

Examination with CE

The Given Diagnostic Imaging System (Yoqueam, Israel) was used in our study. This system is composed of three main subunits: an ingestible capsule endoscope, a data recorder, and a workstation. On the day before the examination, patients were allowed to be on a semi-liquid diet and received complete colonic irrigation (polyethylene glycol 4000, PEG4000) 12 h before the examination. Patients were deprived of water for 4 h and defoamer (simethicone) was administered orally 30 min prior to the examination, respectively. Informed consent was obtained. Four hours after swallowing the capsules, subjects were allowed to take light snacks such as milk. During the examination, subjects were allowed to move freely but exposure to any strong electromagnetic field would be avoided. Eight hours later, data recorded would be downloaded to a RAPID workstation for graphic analysis. All capsules were for single use only.

Data analysis

Findings on CE were classified as diagnostic and non-

Table 1 Baseline characteristics of patients with OGIB (*n* = 309)

Item	All (<i>n</i> = 309)	Massive bleeding (<i>n</i> = 49)	Chronic recurrent overt bleeding (<i>n</i> = 260)
Mean age $\pm$ SD (yr)	55.5 $\pm$ 16.6	52 $\pm$ 19.1	56.1 $\pm$ 16.1
Years (range, yr)	13-87	13-87	15-86
Gender <i>n</i> (%)	150 M (48.5), 159 F (51.5)	27 M (55.1), 22 F (44.9)	123 M (47.3), 137 F (52.7)
Patient status (<i>n</i>)			
Outpatient	141	0	141
Inpatient	168	49	119
Median units of RBCs transfused (U/mL)	4.63 $\pm$ 4.23	8.00 $\pm$ 6.46	4.00 $\pm$ 6.85
Mean Hb value (range, g/dL)	8.73 (4.52-12.10)	6.20 (4.52-9.85)	9.21 (6.56-12.10)

RBCs: Red blood cells; Hb: Hemoglobin; M: Male; F: Female.

diagnostic lesions. Examples of non-diagnostic lesions included red spots, white spots, erythema, scatter lymphangiectasia, active bleeding, blood clots, or small polyps. Examples of diagnostic lesions included angioectasias, tumors, or ulcers, obvious small bowel inflammation, and roundworms. A diagnostic lesion had to definitely or probably account for the patient's bleeding and was diagnosed by two experienced endoscopists.

Statistical analysis

Statistical methods included χ^2 analysis for comparison of the positive diagnostic yield of CE between patients with acute large bleeding and chronic recurrent overt occult GI bleeding. SPSS 16.0 (SPSS Inc. Chicago, IL) was used in our study, and *P* < 0.05 was considered a statistical significance.

RESULTS

Patient baseline characteristics

The 309 cases with OGIB in this study are listed in Table 1 with regard to their baseline characteristics. Prior to CE, for all patients, the cause of OGIB could not be identified with either gastroscopy or colonoscopy. Among them, 39.16% (121/309) underwent a CT scan to detect the source of GI bleeding at the same time, and the percentage of patients who finally underwent surgical operation was 11% (34/309).

Results of CE

Completion of CE: Of all CE procedures performed, 81.88% (253/309) extended through the entire small intestine. However, the rate of going right through the intestine in the chronic recurrent overt bleeding group was higher than that of the massive bleeding group (83.85% *vs* 69.39%, *P* = 0.017, respectively). Four cases with gastric motility disorder resulting in the capsule remaining in the stomach during the entire test were compromised, and another case resulted in emergency surgery because of the large amount of bleeding.

The diagnostic ability of CE: The yield of CE, defined

Table 2 The yield of small bowel diagnostic findings on capsule endoscopy *n* (%)

	All (<i>n</i> = 309)	Massive bleeding (<i>n</i> = 49)	Chronic recurrent overt bleeding (<i>n</i> = 260)
Diagnostic	166 (53.7)	29 (59.2)	137 (52.7)
Non-diagnostic/negative	143 (46.3)	20 (40.8)	123 (47.3)

Table 3 CE diagnostic yields of massive bleeding and chronic recurrent bleeding *n* (%)

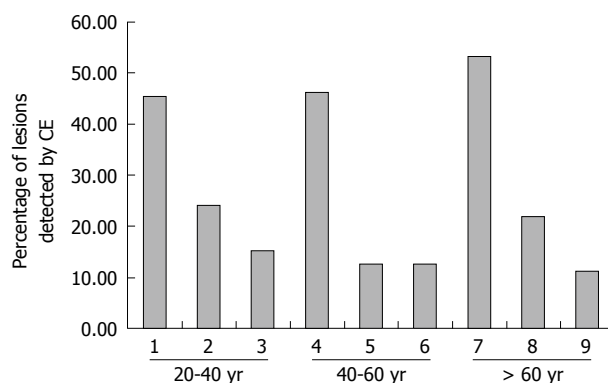
Type of lesion	All	Massive bleeding	Chronic recurrent overt bleeding
Angioectasias (Figure 2)	49 (28.3)	6 (22.2)	43 (29.9)
SB tumor	53 (30.6)	15 (51.7)	38 (26.0)
Mesenchymoma (Figure 3)	15 (8.7)	5 (17.2)	10 (6.9)
Polyps	13 (7.5)	2 (6.9)	11 (7.6)
Hemangioma (Figure 4)	6 (3.5)	2 (6.9)	4 (2.8)
Lipoma	4 (2.3)	0 (0)	4 (2.8)
Lymphoma (Figure 5)	4 (2.3)	2 (6.9)	2 (1.4)
BRBNS	2 (1.2)	2 (6.9)	0 (0)
Adenoma	1 (0.6)	1 (3.5)	0 (0)
Metastatic melanoma	1 (0.6)	0 (0)	1 (1.4)
Mass	7 (4.0)	1 (3.5)	6 (4.2)
Crohn's disease (Figure 6)	25 (14.5)	2 (6.9)	23 (16.0)
Ulcer	15 (8.7)	2 (6.9)	13 (9.0)
Multiple ulcer	11 (6.4)	1 (3.5)	10 (7.6)
Isolated ulcer	4 (2.3)	1 (3.5)	3 (2.1)
SB verminosis	12 (6.9)	2 (6.9)	10 (6.9)
Non-specific enteritis	10 (5.8)	0 (0)	10 (6.9)
SB diverticulum	7 (4.0)	1 (3.5)	6 (4.2)
SAME	1 (0.6)	1 (3.5)	0 (0)
Portal hypertension SB disease	1 (0.6)	0 (0)	1 (1.4)
Total	173	29	144

SAME: Superior mesenteric artery embolus; SB: Small bowel; BRBNS: Blue rubber bleb nevus syndrome.

as diagnostic lesions as the source of OGIB, was 53.72% (166/309) in the entire cohort and slightly higher in those patients with massive bleeding compared to those in the chronic recurrent overt bleeding group, with no significant statistical difference between groups (59.18% *vs* 52.69%, *P* > 0.05, respectively) (Table 2).

Small bowel angioectasias and small bowel tumors (masses) were common and accounted for over 28% of lesions in the entire cohort (Table 3). In the chronic recurrent overt bleeding group, angioectasia incidence was over 29% (Table 3), while in the massive bleeding group small bowel tumors were a common finding at over 51%. Angioectasias were the second most common lesion and accounted for 22.2%, while Crohn's disease was in third place (Table 3).

Age and diagnostic yield of CE: Increasing patient age was associated with positive diagnostic yield of CE and the findings of OGIB were different according to age range. In the 20-40 years age group, Crohn's disease was found in almost 45.5% (15/33) of cases, and in the 40-60 years age group, tumor incidence was 46.2% (26/56). Angioectasias occurred in over 50% (39/73) of cases when the patients' age was over 60 years (Figure 1).

**Figure 1** Lesions detected by capsule endoscopy (CE) for different age ranges. 1: Crohn's disease; 2: SB tumor; 3: Non-specific enteritis; 4: SB tumor; 5: Crohn's disease; 6: Angioectasias; 7: Angioectasias; 8: SB tumor; 9: Ucler.

Failed diagnoses of CE: Among the non-diagnostic studies, 15 cases were diagnosed by further examinations, such as CT scan, angiography, and surgery. In the massive bleeding group, two cases were negative and three cases were actively bleeding with unavailable diagnostic lesions. In the chronic recurrent overt bleeding group, four cases were actively bleeding but without diagnostic lesions. One case was diagnosed as tumor but finally confirmed as Meckel's diverticulum by surgical operation. One case was diagnosed by CE as ulcer and was confirmed as tumor by surgical operation. Four cases were negative.

DISCUSSION

OGIB is the main indicator of the appropriateness of CE as a diagnostic procedure. Many studies have demonstrated a high degree of usefulness of CE as a diagnostic method. The common findings of OGIB reported are angioectasias, small bowel tumor or mass, inflammation, Meckel's diverticulum, *etc.* Barium small bowel examination, angiography and push enteroscopy were the common evaluations for OGIB before the development of CE. Barium showed a diagnostic rate of 3%. Angiography was an invasive method, with a diagnostic rate of 27%-77%, requiring a blood flow > 0.5 mL/min, while push enteroscopy could not reach the lower part of the small bowel and had a diagnostic rate of 19%-32%. Recently, double-balloon enteroscopy (DBE) has been introduced for the diagnosis of OGIB, as reported by a meta analysis^[8]. The diagnostic yield from CE was significantly higher than that of DBE without the combination of oral and anal insertion approaches [137/219 *vs* 110/219, OR 1.67 (95% CI: 1.14-2.44), *P* < 0.01, respectively], but not superior to the yield of DBE with combination of those two insertion approaches [26/48 *vs* 37/48, OR 0.33 (95% CI: 0.05-2.21), *P* > 0.05, respectively]. However, the DBE is invasive and patients are not as tolerant of this method. CE is the only non-invasive method which can examine the whole small intestine.

All cases in our study could tolerate well the CE examination. Five cases were compromised, one in the chronic recurrent overt bleeding group because of capsule

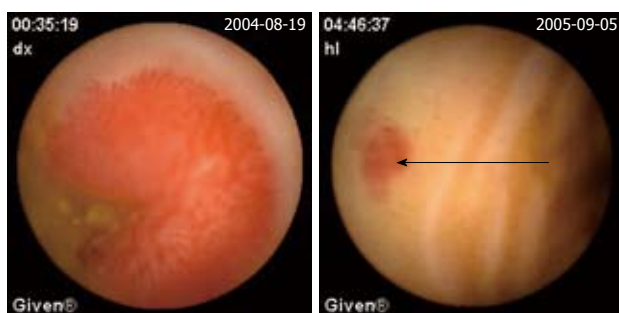


Figure 2 Angioectasias. The red spot is the site of angioectasias (arrow).



Figure 4 Hemangioma. The red-blue bubble is the site of hemangioma (arrow).



Figure 3 Mesenchymoma.

retained in the stomach, and four in the massive bleeding group. Among these four cases, three were compromised because of somatostatin; the capsules were all retained in the stomach due to disorders of gastric mobility. In one Crohn's patient CE was obstructed. No other complication occurred. Therefore, the complication rate was 1.3%. Technical problems and complications of CE are infrequent. Former studies showed a rate of CE complications ranging from 0.75%-5%^[9-11] and also that the existence of patients who had difficulty swallowing the capsule was a rare event. In patients with known altered gastric mobility, the problem could be easily solved by endoscopic placement of the capsule into the duodenum^[12,13].

In our study, CE progressed through the whole small bowel in 81.55% (253/309) of patients, and complete progression rate in the chronic recurrent overt bleeding group was higher than that of the massive bleeding group. CE did not reach the ileocecum because of retention in the intestine due to motility disorder or stricture of the intestinal lumen. Capsule retention occurred almost universally at the site of lesions. In these patients, surgical intervention to retrieve the trapped capsule also allowed for resection of the small bowel involved in the lesion. CE retention by the lesions in our study was found in small bowel Crohn's disease cases and small bowel tumors.

The bowel preparation with PEG and simethicone ensured a good quality of pictures. The 309 patient cohort in our study is the largest series in published studies. The diagnostic rate in our study was 53.72%, which was similar to previous published studies. The diagnostic accuracy yield of CE in published reports ranges from a rate of 30% to 92%, mainly depending on the definition of positive findings and the status of bleeding investigated^[3,5,9,14-16]. The

yield in a series of 260 patients previously reported was 53%. In patients with ongoing obscure overt GI bleeding the yield was 87%, and 46% in patients with obscure occult bleeding^[11]. In this present study we included the presence of active blood as a positive finding. We discovered that the diagnostic rates in both the massive bleeding group and the chronic recurrent overt bleeding group were not statistically significantly different; 59.18% (29/49) and 52.69% (137/260), respectively. These results demonstrate that CE is the first diagnostic option for patients with massive bleeding, since the diagnostic rate was not affected by blood in the small bowel lumen. The relationship between the timing of the procedure and the diagnostic yield of CE remains a controversial issue. Pennazio *et al*^[9] found the highest yield in patients with ongoing GI bleeding, and therefore argued for ordering a CE procedure earlier in the setting of obscure overt bleeding. One would presume that blood in the GI tract would mask visualization of the mucosa and that lesions could easily be missed. Delaying CE until after an acute bleed may allow for clearance of residual blood and better visualization of the mucosa and suspicious lesions. However, our study demonstrated that acute large bleeding did not affect the diagnostic rate.

The definition of a positive finding on CE is an important idea not addressed in early reports^[17]. Although experts have begun to address this issue, final consensus has not yet been reached^[17-19]. For the purposes of this study, nonspecific mucosal changes such as red spots, erythema, scatter lymphangiectasia and thickened folds were considered to be clinically insignificant. Lesions such as angioectasias, tumors, masses or ulcers were included as positive findings in this series if they could completely or partially account for the GI bleeding. In our study, active bleeding but without definite lesion was described as a non-diagnostic finding. Figures 2-6 show the typical lesions found in our study.

There were 15 cases confirmed as being a pseudo-negative result surgically. Five were in the massive bleeding group; 2 of these on CE were negative, and 3 of them found only active bleeding but without diagnostic lesions. Ten cases were in the massive bleeding group, 4 of them on CE were negative, and 4 of them found only active bleeding but without diagnostic lesions. One case was diagnosed as tumor but finally confirmed as Meckel's diverticulum by surgery, while another case indicating an ulcer was confirmed as small tumor by surgery.



Figure 5 Lymphoma.



Figure 6 Crohn's disease.

CE can completely cover the whole GI tract and the procedure is well tolerated. It may provide false-positive and false-negative findings due to the possibility of uncontrollable movement and low-resolution pictures taken^[20]. It was recently reported that CE missed an advanced small intestinal cancer that was later diagnosed with push enteroscopy^[21], suggesting that CE is not an exclusive procedure but one complementary to other diagnostic tools in the assessment of small intestinal lesions. In Mehdizadeh's series^[22], CE found a potential bleeding source in 63 patients, but 34.9% (22/63) of them received negative results in the following DBE procedure. In our study, failed or negative findings of CE may have resulted from four main reasons: (1) growth pattern of lesions: transmural and extra-mural lesions could not be detected by CE; some angioectasias could only be detected by microscope; (2) technical factors of CE: the visual field of CE was only 140°, which perhaps leads to a blind spot; some lesions could be omitted due to movement of CE which could not be controlled. One case with Meckel's diverticulum was diagnosed as small bowel tumor because of only partial views of the lesion; (3) cleanness of small bowel: in our study all cases were prepared with PEG and simethicone prior to examination and the visualization of CE was better than without any preparation, but in some cases, bubbles and food debris still affected the visualization. Delay of gastric motility caused by disease or drugs such as somatostatin would retain the capsule in the stomach, which could not go through the whole small bowel during the working time. The large number of false positives may result from the definition of clinically significant *vs* clinically insignificant findings.

In this study, common findings of lesions with OGIB were small bowel tumor (mass), angioectasias and

Crohn's disease. In the massive bleeding group, common findings of OGIB were small bowel tumor or mass and angioectasias, while in the chronic recurrent overt bleeding group common findings of OGIB were angioectasias, small bowel tumor (mass), and Crohn's disease. A higher positive percentage of small intestine tumors was found compared with all previous studies published due to the definition of diagnostic lesion. Some of the angioectasias discovered in our study cannot be assumed to contribute to the bleeding, and these cases were not included in the positive findings.

In this study, younger patients were more often found to have Crohn's disease, and older patients (older than 60 years) were prone to have angioectasias, while between 40 and 60 years, tumors accounted for a bigger proportion. According to the American Gastroenterological Association (AGA)^[23], younger patients are likely to have small intestinal tumors, Meckel's diverticulum, Dieulafoy's lesion, and Crohn's disease, while older patients (older than 40 years) are prone to bleeding from vascular lesions, which comprise of up to 40% of all causes, and nonsteroidal anti-inflammatory drug-induced small bowel disease. Our finding was somewhat different from the AGA. Thus, in clinical work, patients who are suspected of having Crohn's disease should be examined by CE as early as possible.

In summary, tolerance of administration, higher diagnostic yield, improvement in patient outcome and lower complication rate make CE an important advance in the diagnosis of OGIB. The AGA strongly recommended the CE as the first choice for OGIB. In our large cohort undergoing clinical investigation, we demonstrate that diagnostic rate of OGIB is determined by the definition of a clinically significant lesion. In addition, the massive bleeding status did not affect the positive detection rate.

COMMENTS

Background

Capsule endoscopy (CE) is a tool for visualization of mucosa throughout the entire small bowel. It has already gained a wide clinical acceptance in the evaluation of obscure gastrointestinal bleeding (OGIB). Despite promising preliminary results, published studies were limited to small groups of patients with heterogeneous indications.

Research frontiers

CE is now commonly performed for gastrointestinal bleeding of obscure origin. However, reports about large groups of patients with OGIB receiving CE are rare in the literature up till now.

Innovations and breakthroughs

Published studies of CE in the diagnosis of OGIB were limited to small groups of patients. The study analyzed a large group of OGIB patients, so the finding will be more objective. The authors concluded that the clinically important findings by CE in patients with OGIB were not affected by the different bleeding status, but disease diagnosis by CE is related to different bleeding status and ages. Small bowel tumor was the most common finding in general and also in the massive bleeding group. Angioectasias accounted for the majority of cases in the chronic recurrent overt bleeding group.

Applications

CE is now widely used to diagnose small bowel diseases, especially in patients with OGIB. More and more patients who are suspected of having small bowel diseases are diagnosed using CE with less discomfort, less time taken and also fewer financial costs.

Terminology

CE is a device that can be easily swallowed by patients. It can progress through the whole digestive tract and take two photos every second. These photos can be studied on a workstation.

Peer review

The study is well constructed. It is a retrospective review of all the OGIB cases from a single referral centre and is, admittedly, one of the biggest series of its kind. Other than that though, it simply confirms findings of previous reports in the field.

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CASE REPORT

Complete remission of gastric Burkitt's lymphoma after eradication of *Helicobacter pylori*

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the question of the potential role of *H pylori* in the pathogenesis of some gastric Burkitt's lymphomas, and show the importance of searching for and eradicating the bacteria in combination with conventional chemotherapy regimens.

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Key words: Burkitt's lymphoma; Gastric lymphoma; *Helicobacter pylori*; Therapy

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Abstract

Burkitt's lymphoma is a highly aggressive non-Hodgkin lymphoma, often presenting in extra-nodal sites. It generally has a poor spontaneous outcome and needs aggressive treatment with systemic and intrathecal chemotherapy. Occurrence at the gastric site is rare. We report the case of a 39-year old woman who presented with a prominent ulcerated lesion of the antrum corresponding histologically to a Burkitt's lymphoma associated with *Helicobacter pylori* (*H pylori*) infection. Interphase fluorescence *in situ* hybridization (FISH) demonstrated *c-MYC* gene rearrangement in tumour cells without *BCL2* or *BCL6* gene translocations. Ulcer healing and tumour regression with a complete histological response were obtained 8 wk after *H pylori* eradication. In spite of this complete remission, taking into account the high risk of recurrence, the patient received systemic and intrathecal chemotherapy. Two years later, the patient remained in complete remission. This is the first report of a gastric Burkitt's lymphoma responding to *H pylori* eradication. These findings raise

INTRODUCTION

The stomach is the most common site of extranodal malignant lymphomas, constituting 30%-45% of all extranodal malignant lymphoma and 1%-7% of all gastric malignancies^[1]. The most common gastric lymphomas are extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT), characterized by an infiltration of the mucosa by centrocyte-like B-cells associated with lymphoepithelial lesions, and diffuse large B cell lymphomas (DLBCL)^[2]. This last terminology is commonly used to describe *de novo* DLBCL or DLBCL associated with a low grade component. Burkitt's lymphoma (BL) is a highly aggressive non-Hodgkin lymphoma, often presenting in extranodal sites or as acute leukaemia. The incidence of gastric BL is low compared to other types of gastric lymphomas, although its true incidence is not yet defined^[3].

There is a close link between *Helicobacter pylori* (*H pylori*) and gastric MALT lymphomas and probably also with gastric DLBCL^[4,5]. But the possibility of an association between *H pylori* and gastric BL has only been suggested

in occasional cases in children^[6,7]. Eradication of *H pylori* is now well established as the first line treatment of gastric MALT lymphoma^[8] and reports in the literature show 60%-80% remission rates^[9-11]. In addition, several cases of complete remission of gastric DLBCL after *H pylori* eradication have been reported^[12-16]. On the contrary, BL is known to require aggressive chemotherapy in order to induce remission, with systemic and intrathecal chemotherapy, and the impact of *H pylori* eradication on gastric BL has never been evaluated. We describe the first case of complete remission of a gastric BL in an adult patient following *H pylori* eradication.

CASE REPORT

A 39-year old woman underwent a first gastroscopy in January 2006. She had complained of abdominal pain for 3 mo. She had no previous history of peptic ulcer disease. Examination revealed no palpable mass in the abdomen, ascites or superficial lymphadenopathy. A barium contrast radiography of the stomach showed an important ulcerated lesion of the antrum on the lesser curvature, suggesting malignancy. The patient underwent a gastroscopy that confirmed the ulcer at the angularis, measuring 5 cm × 5 cm (Figure 1A).

Histologic examination of gastric biopsies showed a diffuse infiltration of the mucosa by atypical lymphoid cells with a CD20+, CD3- immunophenotype and a diagnosis of “*de novo* DLBCL” was made initially by the local pathologist. *H pylori* corpuscles were observed histologically using modified Giemsa stain, and the patient was treated for *H pylori* eradication with pantoprazole 40 mg twice daily, amoxicillin 1 g twice daily and clarithromycin 500 mg twice daily for one week. Then she continued pantoprazole 40 mg once daily for three weeks and was referred to our gastroenterology unit four weeks later for further management of her disease. Initial gastric biopsies were reviewed by an expert hematopathologist (CCB) and additional immunostains and molecular studies were performed. The ulcerated mucosa was diffusely infiltrated by monotonous medium-sized lymphoid cells with round nuclei, scarce cytoplasm and starry sky pattern at low magnification (Figure 2A and B). Numerous mitoses were observed. *H pylori* were detected in biopsy specimens. Immunohistochemistry showed that the lymphoid cells were CD20+, CD5-, CD10+, BCL2-, BCL6+, MUM1-, P53+ and 100% of tumour cells were Ki67 positive (Figure 2C and D, and Figure 3A-C). *In situ* hybridization using EBERs probes was performed and EBV transcripts were not detected in tumour cells. Interphase fluorescence *in situ* hybridization (FISH) analysis was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections using split signal FISH DNA probes for *c-MYC*, *BCL2* and *BCL6* (Dako AS, Glostrup, Denmark) according to the manufacturer's recommendations (www.euro.fish.org). *C-MYC* gene rearrangement was observed in tumour cells without *BCL2* or *BCL6* gene rearrangement (Figure 3D). All these findings were in accordance with the diagnosis of BL.

Follow up endoscopy performed 6 wk after initiation

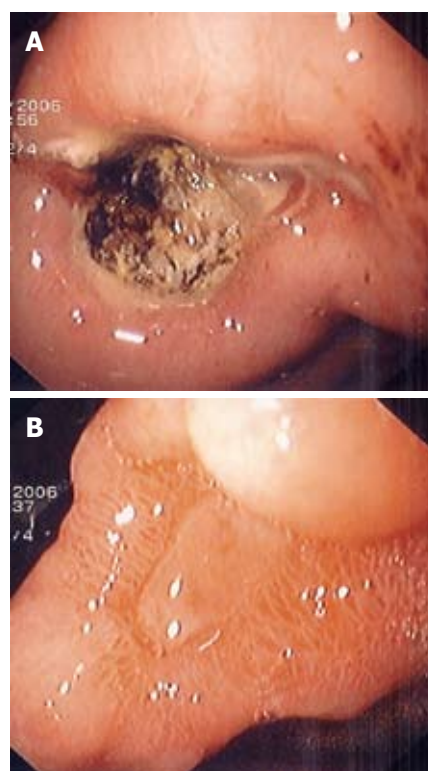


Figure 1 Endoscopic aspect of the gastric lesion before and after *Helicobacter pylori* (*H pylori*) eradication. A: Oesophagogastrroduodenoscopy shows a large ulcer at the angularis; B: Ulcer healing 3 mo after *H pylori* eradication.

of *H pylori* eradication therapy showed the ulcer size had decreased, measuring 3 cm in diameter, and histological analysis of gastric biopsies showed complete remission of the disease and absence of *H pylori*. Endoscopic ultrasonography showed a markedly increased thickness of the gastric wall (10 mm) with disappearance of the layer structure on the lesser curvature of the antrum and with a perigastric lymphadenopathy measuring 7 mm in diameter.

Appropriate investigations were performed to stage the lymphoma. Blood cell counts and biochemical parameters, including LDH and β 2microglobulin level, and hepatic biology were normal. An EBV serology test showed IgG positive and IgM negative viral capsid antigen (VCA) and Epstein-Barr nuclear antigen (EBNA), reminiscent of “an old infection”. Human immunodeficiency virus serology was negative. Bone marrow biopsy and lumbar puncture were normal. Abdominal computed tomography showed an important increase in gastric wall thickness. As a perigastric lymphadenopathy was present at endoscopic ultrasonography, the patient was classified as stage IIE according to the Ann Arbor classification. The International Prognostic Index, including age (older or not than 60 years), LDH level (normal or elevated), tumour stage (I, II/III, IV), number of extranodal sites involved by disease (0 or 1/more than 1), patient performance status (less or more than 2), was scored 1 (low-risk group).

A new endoscopy with biopsy was performed one month later and showed complete healing of the ulcer (Figure 1B), and persistent histological remission of the disease. In spite of the absence of residual disease, taking

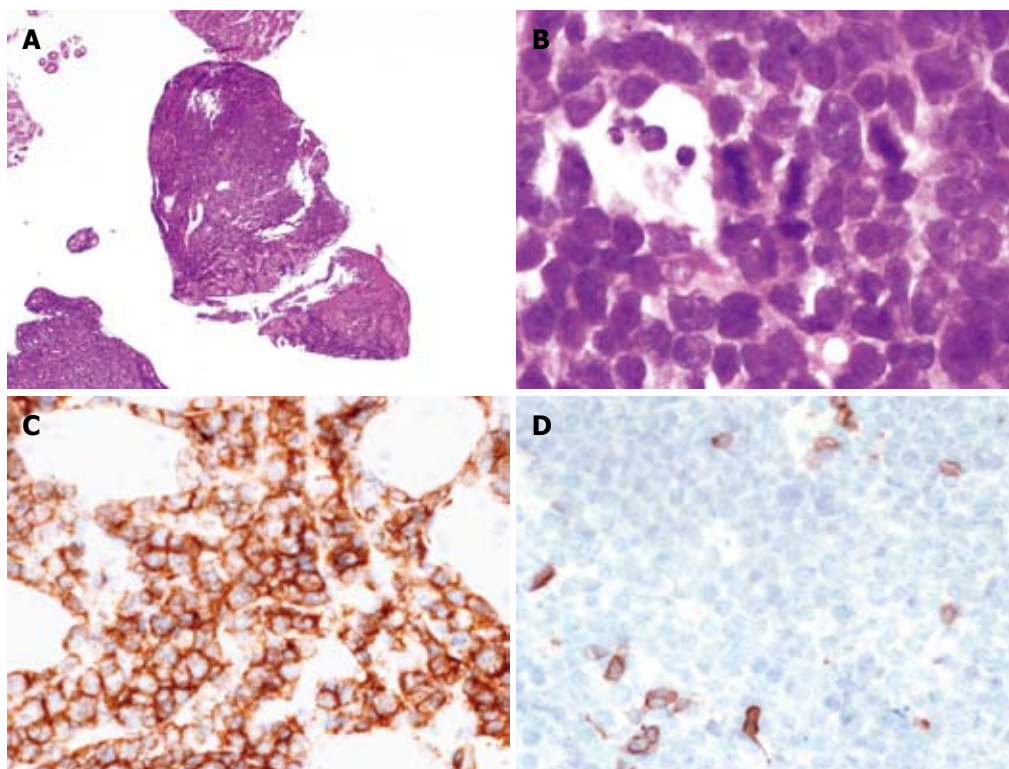


Figure 2 Morphological and immunohistochemical features of the disease. A: At low magnification, the gastric mucosa is diffusely infiltrated by medium sized lymphoid cells (HE, $\times 25$); B: Tumour cells are medium sized with scarce cytoplasm, round nuclei and fine chromatin; numerous mitoses are observed; tangible body macrophages are present (HE, $\times 1000$); C: Immunohistochemistry shows that the tumour cells are CD20+, $\times 400$; D: CD5-, $\times 400$.

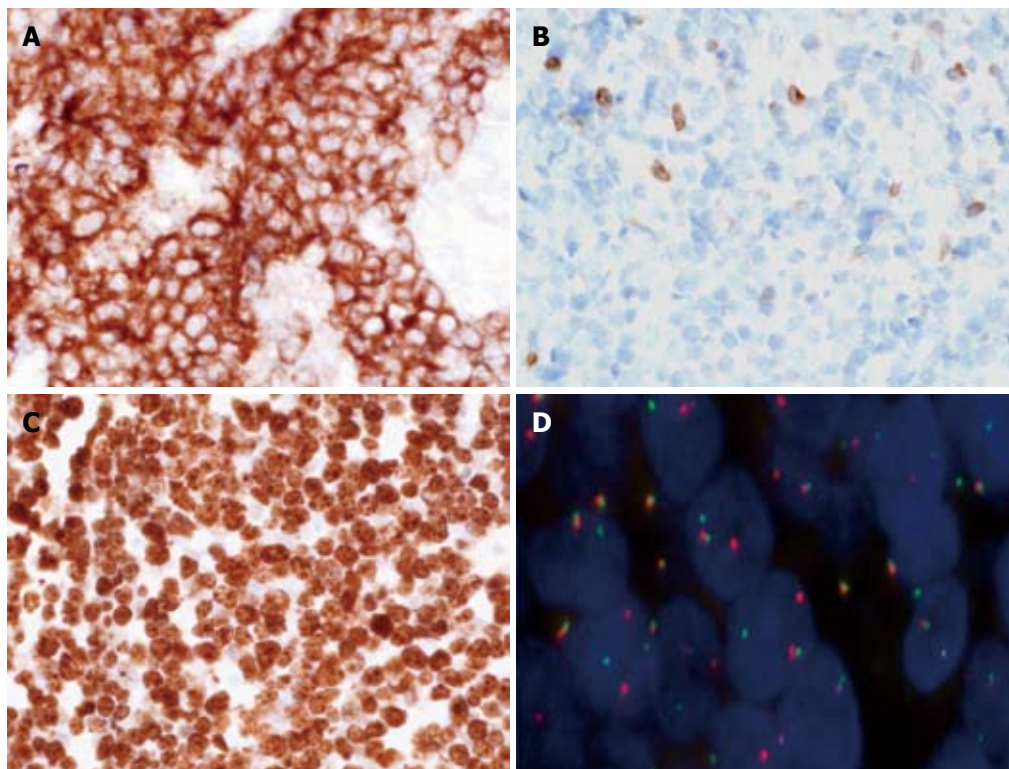


Figure 3 Immunohistochemical and cytogenetic features of the disease. A: Tumour cells are CD10+, $\times 400$; B: BCL2-, $\times 400$; C: 100% of tumour cells are Ki67+, $\times 400$; D: Fluorescence *in situ* hybridization (FISH) performed with split signal FISH DNA probes for *c-MYC* revealed distinct red and green split signals in many nuclei, consistent with *c-MYC* gene rearrangement, beside yellow fusion signals for the non-rearranged alleles, $\times 1000$.

into account the high risk of lymphoma recurrence, she then received systemic chemotherapy, which consisted of

cyclophosphamide, vincristine, prednisone, doxorubicin and methotrexate, associated with intrathecal injection of

methotrexate, until August 2006. The gastric endoscopic aspect with systematic biopsies was checked every 6 mo. The last outcome visit, in March 2008, with a new scanography and a new endoscopy and endoscopic ultrasonography confirmed the complete remission of the disease.

DISCUSSION

BL is a mature aggressive B-cell lymphoma, characterized by a high degree of proliferation of the neoplastic cells and deregulation of the *c-MYC* gene^[17,18]. It is a distinct entity that occurs sporadically worldwide, sometimes associated with immunodeficiency or immunosuppression, and is endemic in central Africa (the lymphoma belt of Africa). Cells in both sporadic and endemic forms of BL are B lymphocytes that have clonal immunoglobulin (*IG*) gene rearrangement and 8q24/*MYC* translocation to the *IG* heavy chain region, 14q32 or less commonly at the lambda 22q11 or kappa 2p12 light chain loci. Epstein Barr virus and malaria are recognised as important cofactors of endemic BL. A combination of these two diseases seems to boost the incidence of endemic BL in the lymphoma belt by a factor of 100-150. But this disease can occur in the absence of both of these infections and arboviruses and plant tumour promoters are other possible local cofactors. There are probably other cofactors which are unknown.

The stomach is the most common site of extranodal malignant lymphomas. The incidence of gastric BL is low as compared to other types of gastric lymphomas, although its true incidence is not yet defined. Like other types of BL, gastric BL is known to have a highly aggressive clinical course. However, one study including 21 patients with a gastric BL, showed a better outcome for these patients as compared to 26 non-gastric BL with a long-term survival of 90%. An especially good outcome was observed when the disease was localized to the stomach and the authors concluded that, in this case, prognosis of BL and DLBCL could be similar. This observation suggested a common pathogenesis^[3].

There is a close association between *H pylori* and gastric lymphomas^[4]. But the link between *H pylori* and BL has not been demonstrated yet. *H pylori* plays an important role in the development and growth of MALT lymphoma and its eradication has been shown to achieve durable regression in 56% to 100% of patients with early stage low-grade gastric MALT lymphoma^[8-11]. *In vitro* co-culture of *H pylori* with tumour B cells in the presence of non-neoplastic T cells proved that the bacteria were able to induce proliferative responses of MALT lymphocytes but not large B cells^[19]. Therefore, DLBCL of the stomach were generally believed to be *H pylori*-independent, and unlikely to respond to antibiotic therapy. However, in addition to sporadic cases, one prospective study of 24 patients with DLBCL has shown that antibiotic therapy could also result in complete remission in patients with early stage gastric DLBCL with low grade components^[15]. The same authors compared two prospective studies of the efficacy of *H pylori* eradication

in the treatment of early-stage MALT lymphoma and DLBCL of the stomach and showed that the therapeutic efficacy of antibiotics for stage IE gastric high grade transformed MALT lymphoma was similar to their efficacy in stage IE low-grade lymphoma. It was suggested that the response was possible only in DLBCL with low grade component and more frequent in cases of superficial gastric wall involvement and in the absence of BCL10 protein nuclear expression^[16-20].

Histologically, the distinction between diffuse large B cell lymphoma and BL is sometimes difficult, especially in adult patients. It is well known that these two entities may show overlapping morphological and immunohistochemical features^[21,22]. Whether this case represents "a B cell lymphoma, unclassifiable, with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma", a provisional entity defined in the recent 2008 World Health Organization (WHO) classification of lymphoid neoplasms may be discussed^[2]. However, this case met all the characteristic morphological (starry sky appearance, monotonous medium sized tumour cells with round nuclei and scarce cytoplasm), immunohistochemical (CD10+, BCL2-, 100% Ki67 positivity) and cytogenetic (*MYC* translocation without *BCL2* or *BCL6* translocation) diagnosis criteria of BL according to the 2008 WHO classification, therefore excluding the hypothesis of *de novo* DLBCL.

In the present case, eradication of *H pylori* was performed before the definitive histological results were available. So, we had the opportunity to observe the first case of BL remission following *H pylori* eradication in an adult patient. This observation highlights the potential role of an infectious agent, notably *H pylori*, as a cofactor in the development of BL and suggests that *H pylori* eradication should be systematic in the context of *H pylori* positive gastric BL in association with conventional chemotherapy regimens.

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A case of asymptomatic fungal and bacterial colonization of an intragastric balloon

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Abstract

Intragastric balloon therapy, as a part of a multidisciplinary weight management program, is an effective short-term intervention for weight loss. Although the insertion procedure is easy and generally well tolerated by patients, a few complications can occur. We report here a heavy smoker with intragastric balloon insertion complicated by colonization with opportunistic organisms. The 27-year-old female, body mass index 35.5 kg/m², had a BioEnterics® Intragastric Balloon inserted under conscious sedation without any peri-operative complications. Six months later, when the standard removal time arrived, the balloon was seen to be covered with a necrotic white-gray material. Microbiological examination revealed *Enterobacter cloacae* and *Candida* species yeast colonies. We recommend that asymptomatic fungal and/or bacterial colonization should be considered among the complications of the intragastric balloon procedure, despite its rarity.

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Key words: Gastric balloon; Obesity; Complications; Infection

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Coskun H, Bozkurt S. A case of asymptomatic fungal and bacterial colonization of an intragastric balloon. *World J Gastroenterol* 2009; 15(45): 5751-5753 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5751.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5751>

INTRODUCTION

Obesity will be one of the 21st century's most important problems and will become a worldwide epidemic. In 2015 approximately 2.3 billion people will be overweight and 700 million people will be reported to be obese^[1]. Obesity treatment options consist of a calorie-restricted diet, lifestyle modification, medical treatment, endoscopic intragastric balloon application and bariatric surgery^[2]. In 1982, the intragastric balloon was used for the first time by Nieben *et al*^[3], who observed that long-lasting and well-tolerated gastric bezoars can result in significant weight loss.

Intragastric balloon therapy, as a part of a multidisciplinary weight management program is an effective short-term intervention for weight loss. In experienced hands the application and removal of the intragastric balloon is easy and has relatively low morbidity and mortality compared to bariatric surgery^[4,5].

The major complications of the intragastric balloon are intolerance, gastric ulcers, gastric erosion and esophagitis, spontaneous deflation of the balloon, ongoing vomiting for 1-3 wk or more, abdominal pain and gastroesophageal reflux. There are also a few reported cases of gastric perforation, dilatation and small intestinal obstruction^[6,7].

We present here an asymptomatic intragastric balloon infected by *Candida* and *Enterobacter* species which has not been encountered previously in the literature, and we discuss possible and probable causes.

CASE REPORT

A 27-year-old female, heavily smoking patient, 91 kg in weight and body mass index (BMI) 35.5 kg/m² had a BioEnterics® Intragastric Balloon (BIB; BioEnterics Corporation, California, USA, S/N: 123456789) inserted. The patient was premedicated with 0.05 mg/kg iv midazolam (Dormicum Roche, Turkey) for 10 min before the insertion and an additional 0.5-0.75 mg/kg

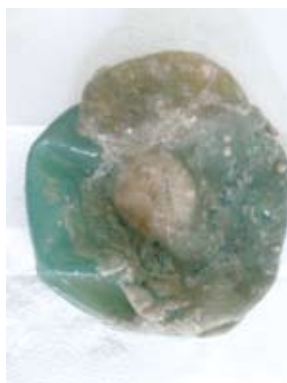


Figure 1 Removed silicon balloon covered with a necrotic white-gray material.

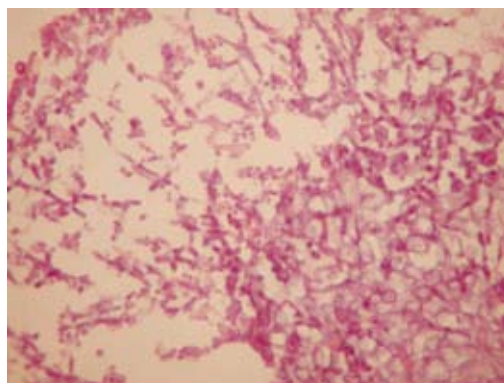


Figure 2 Necrotic material with mycotic hyphae and spores (HE, × 600).

propofol (Diprivan, Astra Zeneca, Turkey) was given iv during the application to attain deeper sedation^[8]. Under video-endoscopic view, the balloon was positioned in the stomach and inflated with 550 mL NaCl 0.09% mixed with 10 mL methylene blue solution. The upper gastrointestinal endoscopic findings were normal. As is common, the patient experienced intense nausea, vomiting and cramping abdominal pain in the first 48 h. With appropriate medical support these standard complaints subsided significantly by the fifth day after the procedure. In the following days the patient was given a 1200 kcal/d diet. The balloon was removed according to protocol on the 180th d after placement. On the 180th d, the patient's weight was 69 kg, with BMI 26.9 kg/m², excess weight loss 62.8%, and excess BMI loss 81.4%. During the removal of the balloon, endoscopy revealed normal esophageal and gastric mucosal surfaces except slight gastritis. However, the surface of the silicon balloon was covered with a necrotic white-gray layer (Figure 1). This necrotic material covered almost 80% of the total surface of the balloon. The balloon had been removed endoscopically with no problems. Histopathologic examination revealed a few fungal hyphae, polymorphonuclear infiltration of leukocytes and mononuclear cells (Figure 2), with active and chronic inflammation. Gram staining showed epithelial cells, leukocytes, gram-negative bacilli and yeast cells. With Ziehl-Nielsen staining, acid-resistant bacilli were not seen. Microbiologic examination revealed *Enterobacter cloacae* and *Candida* species yeast colonies. The patient was discharged with appropriate non-specific supportive treatment without any clinical problems. The patient was evaluated endoscopically after a 3-mo clinical follow-up and no pathology was found.

DISCUSSION

Intragastric balloon application has relatively low morbidity and mortality compared to other bariatric procedures. In particular it is the primary choice for excessively morbidly obese patients who are prepared to undergo surgical bariatric procedures.

The complication rate is extremely variable between studies. This is because minor complications can easily be managed by phone calls or during scheduled visits. The major complications of the intragastric balloon procedure are balloon intolerance (7%), gastric ulcer (0.4%),

gastric erosion and esophagitis (18.2%), spontaneous deflation of the balloon (3%-23%), ongoing vomiting for 1-3 wk or more (0%-15.9%), abdominal pain (5%), and gastroesophageal reflux (1.8%). There are also a few reported cases of gastric perforation (0.1%-0.21%), dilatation and small intestinal obstructions (0.17%-0.8%)^[5-7]. Infection arising from intragastric balloon insertion is not a common issue. There is no such published case in the literature.

The isolated microorganism, *Enterobacter cloacae* is a gram-negative bacillus. Frequently, it causes nosocomial infections in patients in whom respiratory support instruments or catheterizations are applied, or it appears as opportunistic infections in patients with immunodeficient and debilitating diseases^[9].

Candida is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a constituent of the normal flora of the skin, mouth, vagina, and stool^[10].

Colonization of soft lining materials with microorganisms, particularly *Candida* species, is a common clinical problem. The intragastric balloon has a silicon elastomer structure and da Silveira *et al*^[11] mentioned in their paper that silicone-based soft lining materials are more susceptible to *Candida* adhesion.

Candidiasis of the gastrointestinal system mostly affects the esophagus and the infection is seen as patch plaques on the mucosa^[12]. Although a typical fungal infection was not detected in the esophagus of our patient, such contamination can not be excluded and may have occurred during endoscopic intervention, i.e. the balloon passage through the oral cavity or during its application.

Of the predisposing factors causing gastric candidiasis, delayed gastric emptying and gastric stasis have been proposed. It is a well known issue that one of the mechanisms of action of the intragastric balloon is to delay gastric emptying^[13]. Thus the slowing down of normal peristalsis of the stomach by the intragastric balloon may allow opportunistic organisms to colonize more readily.

The patients in whom an intragastric balloon is inserted should take proton pump inhibitors during application of the balloon because of gastric hyperacidity. These proton

pump inhibitors result in a hypochlorhydric gastric medium which makes opportunistic infections more likely^[14-17].

The important feature identified in the present case was that she was a heavy smoker. It is known that cigarette smoke has many effects on the gastric mucosa. These can result in progression from gastritis to gastric ulcers and even carcinoma. Smokers appear to be at higher risk of becoming infected with *Helicobacter pylori* (*H. pylori*) and this increased risk may result from the adverse effects of smoking on antioxidants or the immune system which may interfere with normal protection against *H. pylori*^[18-20].

Because the infection detected on the balloon was a colonization rather than a systemic infection we recommended appropriate non-specific supportive treatment for the patient. However, we recommend systemic antifungal and antibacterial treatment for patients in whom the gastrointestinal integrity is damaged, who are immunocompromised or who are scheduled for bariatric surgery.

We present here a heavy smoker with an intragastric balloon infection. In our series of 201 endoscopic removals of 263 intragastric balloons, this case was the only one with such an infection. The rare incidence of colonization leads us to believe that this kind of infection may be multifactorial, and no single cause can be highlighted. In patients who receive an intragastric balloon, predisposing factors for opportunistic infections such as gastric stasis, smoking and antacid medications should be taken into consideration and patients monitored for these infections. Any asymptomatic balloon infection, especially opportunistic pathogens, should be treated with appropriate medications if the integrity of the gastrointestinal system is damaged.

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LETTERS TO THE EDITOR

Spontaneous bacterial peritonitis: Few additional points

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Abstract

Spontaneous bacterial peritonitis (SBP) is a treatable complication of decompensated cirrhosis. Coagulopathy with evidence of hyperfibrinolysis or clinically evident disseminated intravascular coagulation precludes paracentesis. Alcoholic hepatitis with fever, leucocytosis and abdominal pain should be evaluated for SBP. Oral ofloxacin is as effective as parenteral cefotaxime in treatment of SBP except for inpatients with vomiting, encephalopathy, or renal failure. Albumin is superior to hydroxyethyl starch in treatment of SBP.

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Key words: Spontaneous bacterial peritonitis; Albumin; Antibiotics

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TO THE EDITOR

We read with great interest the article "Spontaneous bacterial peritonitis" by Koulaouzidis *et al*^[1] in March 7, 2009 issue of *World Journal of Gastroenterology*. The review was extensive. However, some points may be useful for the management of patients with spontaneous bacterial peritonitis (SBP).

Serious complications unusually occur in paracentesis, but a subgroup of patients with renal failure should be carefully monitored. Pache *et al*^[2] observed 9 bleeding complications in their study of 4729 paracentesis patients, which were probably due to qualitative platelet abnormality in 8 patients with renal failure.

Coagulopathy precludes paracentesis only when there is clinically evident hyperfibrinolysis or disseminated intravascular coagulation^[3]. Ascitic fluid leak post paracentesis can be prevented by inserting a needle along the Z-track and keeping the patient in the right lateral position for a few hours.

Biochemical tests are required for total protein and albumin in each ascitic fluid sample whereas optional tests are required for glucose and lactate dehydrogenase levels^[3].

Ascitic fluid culture should be performed before antibiotics are used because even a single dose of antibiotics causes the culture to produce no growth of bacteria in 86% of cases^[4].

Patients with alcoholic hepatitis present with fever, leukocytosis and abdominal pain mimicking SBP. An elevated ascitic fluid polymorphonuclear count in these patients is not due to peripheral leukocytosis^[5] but may represent SBP. Empiric treatment with antibiotics can be discontinued after 48 h if ascitic fluid, blood and urine cultures are negative.

Oral ofloxacin (400 mg, twice a day for an average of 8 d) is as effective as parenteral cefotaxime against SBP when the patients do not have vomiting, shock, grade II (or higher) hepatic encephalopathy or serum creatinine > 3 mg/dL^[6].

Sigal *et al*^[7] have shown in their study that albumin should be used in SBP patients with their serum creatinine > 1 mg/dL, blood urea nitrogen > 30 mg/dL, or total bilirubin > 4 mg/dL, and not used in patients without such indications. Albumin is superior to hydroxyethyl starch in treatment of SBP^[8].

Fernández *et al*^[9] in their randomized trial have shown that daily norfloxacin can prevent SBP and hepatorenal syndrome and has a survival advantage in patients with their ascitic fluid protein < 1.5 gm/dL and at least with one of the following indications, namely serum creatinine $\geq$ 1.2 mg/dL, blood urea nitrogen $\geq$ 25 mg/dL, serum sodium $\leq$ 130 mg/L, Child-Pugh $\geq$ 9 points, and bilirubin $\geq$ 3 mg/dL.

Intermittent dosing of double, enforced trimethoprim-sulfamethoxazole (5 doses per week) or ciprofloxacin (single oral dose of 750 mg per week) may be ineffective against resistant flora^[10].

In conclusion, proper selection of patients for paracentesis, high index for suspected SBP in alcoholic hepatitis patients and albumin treatment can help the management of SBP.

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Meetings

Events Calendar 2009

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

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Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
<http://www.apasl2009hongkong.org/en/home.aspx>

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Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

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Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
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13th International Symposium on Viral Hepatitis and Liver Disease

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Email: bsg@mailbox.ulcc.ac.uk

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2009 Hepatotoxicity Special Interest Group Meeting

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Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
<http://www.easl.ch/>

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Digestive Disease Week 2009

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Orlando, Florida
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www.asco.org/annualmeeting

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Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
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June 20-26, 2009
Flims, Switzerland
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June 24-27 2009
Barcelona, Spain
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www.worldgicancer.com

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Molecular Biology in Clinical Oncology

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Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

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Practical Solutions for Successful Management
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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

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Write as mean $\pm$ SD or mean $\pm$ SE.

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