

RESEARCH COMMUNICATION

***Helicobacter bilis* in Human Gallbladder Cancer: Results of a Case-control Study and a Meta-analysis**

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Abstract

Introduction: Gallbladder cancer is an uncommon neoplasm of uncertain etiology and poor survival. Recently, interest has been generated in bacterial infections and cancers. *Helicobacter* is one such bacterium found to be associated with gastric MALToma, gastric adenocarcinoma and hepatobiliary neoplasms. **Patients and methods:** Fifty four gallbladder cancer and 55 controls with cholelithiasis were studied. *Helicobacter bilis* was identified using 16S rRNA PCR. Relative risk and odds ratio with 95% CI were estimated. A detailed search of literature was carried out and selected relevant articles were extracted. A meta analysis was carried out using a random effect model. **Results:** *Helicobacter bilis* was identified in 32/54 patients and 32/55 controls, The relative risk of gallbladder cancer in *H. bilis* positive cases was 1.05 (95% CI 0.49 to 2.24). Of the 10 identified case control studies on *Helicobacter* in the hepatobiliary tract 3 each were on gallbladder cancer and *H. bilis*. In meta analysis a pooled odds ratio of 4.13 (95% CI 2.68-6.36) favoring *Helicobacter* was observed. Pooled analysis of published studies on gallbladder cancer showed an odds ratio of 1.24 (95% CI 0.63-2.44). **Conclusions:** The present study failed to demonstrate any increase in risk of gallbladder cancer in presence of *Helicobacter bilis*. It may be hypothesized that increased risk observed in earlier studies may be indirectly due to increase in the risk of gallstones, although lack of any study specifically looking at this aspect and absence of normal controls in the present study makes this assumption superfluous.

Keywords: Gallbladder cancer - *Helicobacter bilis* - case-control study - meta-analysis

Asian Pacific J Cancer Prev, 11, 343-347

Introduction

Gallbladder carcinoma (GBC) is an uncommon but fatal disease whose initial symptoms frequently mimic that of gallstone or acid peptic dyspepsia (Scott et al., 1999). Gallbladder cancer is the commonest cancer among females in eastern Uttar Pradesh province of India, and is third commonest cancer of the gastrointestinal tract in developed countries (Pandey et al., 2001). The etiology of gallbladder cancer is still obscure. Studies have shown colonization of hepatobiliary tract by *Helicobacter* species, and have reported to its association with benign as well as malignant hepatobiliary diseases (Fox et al., 1998; Pandey, 2007; Vivekanandan and Torbenson, 2008). In animal models *Helicobacter* sp. causes hepatitis, hepatocellular carcinoma and bacteremia in immunocompromised as well as immuno-competent hosts (Pisani et al., 2006).

Helicobacter pylori is the most studied species. The most recent species identified in humans is *H. bilis*. It was discovered in the bile and gallbladder tissue of the patients affected with cholecystitis in the region with the higher incidence of gallbladder cancer (Fox et al., 1998; Pisani et al., 2008). Presence of the DNA of *H. bilis* and some other species of *Helicobacter* have been identified

in neoplastic and normal liver tissue of cancerous patients and in malignant intra and extrahepatic biliary diseases (Apostolov et al., 2005; Kobayashi et al., 2005; De et al., 2009).

The relation of *Helicobacter bilis* has not been investigated thoroughly with only two previous studies looking at this bacterium in biliary tract cancers. This case control study was carried out to estimate the prevalence of *Helicobacter bilis* in gallbladder tissue of patients with carcinoma of the gallbladder and to compare it with prevalence in gallstone controls using PCR.

Materials and Methods

A total 54 cases of gallbladder cancer and 55 patients with cholelithiasis were included in this study. Cytologically proven cases of gallbladder cancer and ultrasonographically proven cases of cholelithiasis were included in the study. Gallbladder tissue was collected at the time of surgery and was stored at -80°C immediately after the collection. Demographic, clinical and detailed hematological and biochemical data was collected on preset pro-forma.

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Table 1. Sequences of Primers, their Gene Specificity and Cycle Program

S. N.	16Sr RNA gene primer	Nucleotide Sequence	PCR Program	Size
1	HB16Sf	5' GACTAGACTTAGT GTCTGTCGCAC 3'	30 cycles, 1005.bp Tm= 610C	
	HB16Sr	5' TCCGCCTCCT TCCACTCC 3'	30 cycles, 1005.bp Tm= 610C	

Gradient program MJ Mini gradient thermal cycler (Bio-Rad™):

95.0°C	7 minutes	
94 °C	30 seconds	
61°C - 54°C	30 seconds	
72°C	1.50 minutes	2=29
72°C	7 minutes	
4.0	Pause	

Molecular identification

Source of conserved gene identification of *H. bilis* and design of the primer. The bacterium *H. bilis* genome was obtained from NCBI Gene Bank. The Accession Number U18766 has been targeted to *H. bilis* species specific 16S rRNA gene. For the amplification and molecular identification of bacterium primers were designed for conserved stretch of nucleotide in ribosomal activity associated gene ORF 16S rRNA. The designed primers (Table 1) were analyzed on Clustal W2 version with three close bacteria viz. *H. acinonychis*, *H. pylori* and *C. jejuni*, to check their E value and specificity. The 16S rRNA gene primer showed 100 % specificity with *H. bilis*. The primers synthesis was done by Metabion International Deutschland in 100.0µM concentrations and 0.02µmol synthetic scales. The primers amplification located between 299 bp to 1304 (1005.bp) (<http://www.ncbi.nlm.gov/BLAST>). The sequence and their amplification program are given in Table 1.

DNA isolation from tissue sample and bacterial colonies

The homogenized tissue sample was incubated with 1 mg lysozyme at 37°C for 60 minutes. Then 1 ml of 0.1% Triton-X and 5 µl Proteinase-K were added and 30µl of SDS and incubated again at 65°C for 120 minutes. To this, equal volume of Chloroform: IAA (24:1) was added and mixed by vortexing for 15 minutes, centrifuged at 10,000 rpm for 10 minutes and aqueous phase was collected. Then 140 µl of Phenol: Chloroform: IAA (25:24:1) was added and mixed by vortexing for 15 seconds. To the aqueous phase equal volume of Isopropanol was added. The solution was kept at room temperature for 5 minutes, centrifuged at 10,000 rpm for 10 minutes and supernatant decanted. The pellet was washed by 200 µl 70% Ethanol and centrifuged at 10,000 rpm for 10 minutes. The pellets were dried over at 37°C for 30 minutes. Then the pellets were re-dissolved in 50 µl in TE buffer.

PCR amplification

The PCR reaction mix was placed in the well for gradient PCR, the details of the program used are given in Table 1.

Gel electrophoresis of PCR products (amplicons)

To check the amplification after PCR agarose gel electrophoresis was performed with 1% agarose gel in 1x TAE buffer, EtBr was added to make the final concentration of 0.5mg/ml. Gel slab were prepared by the pouring the dissolved agarose in the required plate. Sample with gel loading buffer (0.025% bromophenol blue and xylene cyanol, 10mM EDTA pH 8.0, 50% glycerol) was loaded in the sample well and electrophoresis was carried out at 80 volts. DNA bands were visualized under short wave length UV transilluminator and photographed in Alpha imager EC (Alpha Innotech).

Literature search strategy

A detailed search of Medline, Pubmed, Scopus and Google Scholar was made, the search strategy for Medline being described below. The search was limited to humans. #1 tumor /tumour/neoplasia/cancer/carcinoma; #2 gallbladder /gall bladder/liver/"extrahepatic bile duct"/hepatobiliary#3 cholangiocarcinoma/"hepatocellular carcinoma" /"Klatskin tumor";#4 Helicobacter;#5 #2/#3; #6 #1 and #4 and #5

The abstracts of the articles were read and all articles on benign diseases, observational or single group studies were then excluded. Full articles for the case-control studies were extracted.

Data extraction

From the case control studies the data on methods of detection, number of cases and controls and number of positive cases and controls was extracted along with site of the cancer and type of sample (bile or tissue) examined.

Statistical analysis

Statistical analysis was carried out by using relative risk of gallbladder cancer in presence of *H. bilis*. A meta analysis was carried out to calculate the pooled odds ratio (95% confidence interval) by combining the present data with previous 10 published studies on *Helicobacter* sp in biliary tract malignancies (Fox et al., 1998; Avenaud et al., 2000; Nilsson et al., 2000; Bulajic et al., 2002; Fukuda et al., 2002; Matsukura et al., 2002; Huang et al., 2004; Murata et al., 2004; Kobayashi et al., 2005; Bohr et al., 2007) and three earlier studies on *H. bilis* (Fox et al., 1998; Matsukura et al., 2002; Murata et al., 2004). A random effect model was used. Heterogeneity of the studies was tested with chi square test.

Results**Demography**

The mean age of the cases was 53.6+11.9 years and of controls was 49.1+15 years (P=0.08). There were 36 females and 19 males in the control group and 34 females and 20 males (p=0.52) in the carcinoma group. All of the patients with cancer had either locally advanced operable gallbladder cancer or had inoperable disease, 23 being jaundiced.

Results of PCR

A total of 32/54 cancer patients and 32/55 controls were

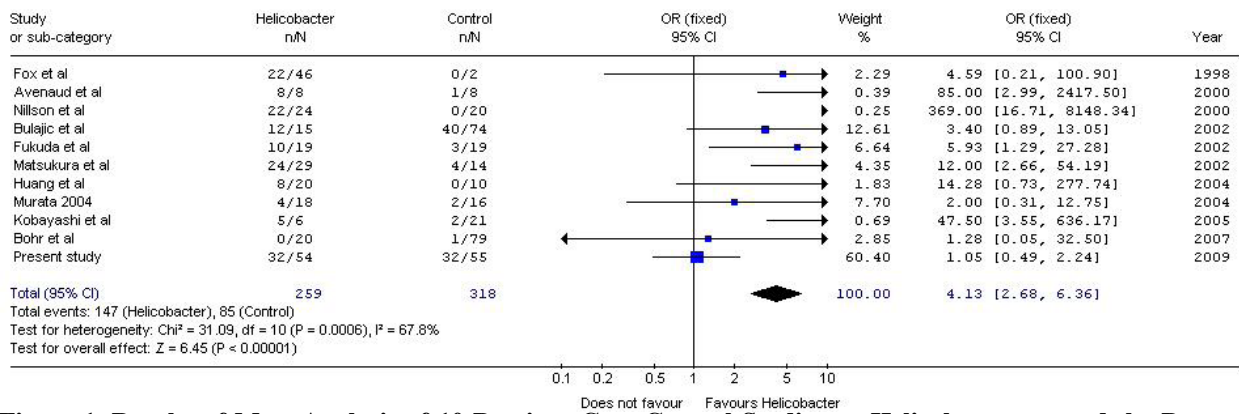


Figure 1. Results of Meta Analysis of 10 Previous Case Control Studies on Helicobacter sp. and the Present Study on Hepatobiliary Tract Malignancies using Random Effect Model

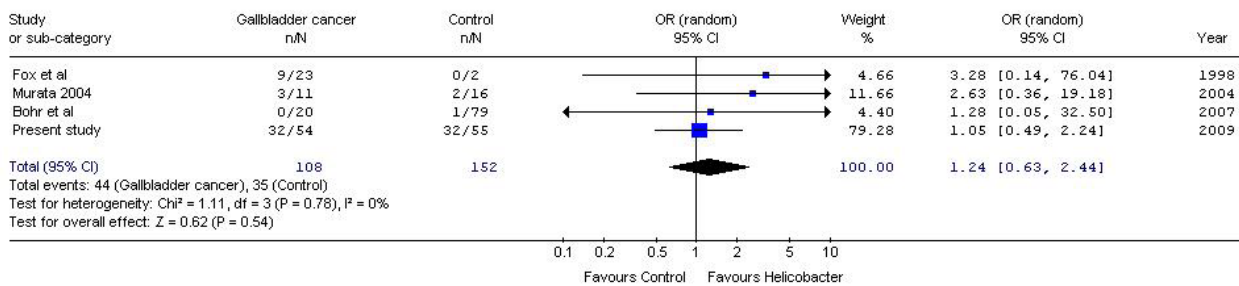


Figure 2. Results of Meta Analysis of 3 Previous Case Control Studies on Helicobacter sp. and the Present Study on Gallbladder Cancer using Random Effect Model

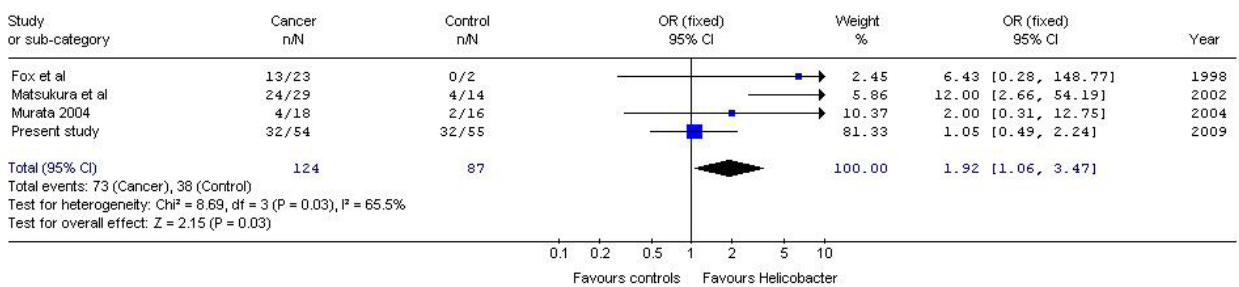


Figure 3. Results of Meta Analysis of 3 Previous Case Control Studies and the Present Study of Helicobacter Bilis on Hepatobiliary Tract Malignancies using Random Effect Model

positive for *H. bilis*. The relative risk of gallbladder cancer in *H. bilis* positive cases was 1.05 (95% CI 0.49 to 2.24).

Meta-analysis

When the result of present study were added to the meta analysis of earlier 10 studies looking at association of Helicobacter sp on biliary tract cancers, an pooled odds ratio of 4.13 (95% CI 2.68-6.36) favoring *Helicobacter* was observed (Figure 1). 147/249 cases and 85/318 controls were positive for *Helicobacter* sp. However, the heterogeneity among the studies was very high (chi square 31, p=0.0006). The present study had the highest weight (60%). Of the earlier 10, studies only 3 were on gallbladder cancer. On pooling the results of the present study with these an odds ratio of 1.24 was observed (95% CI 0.63-2.44) (Figure 2). Of the 108 cases of gallbladder cancer studied 44 were positive for *Helicobacter* compared with 35/152 controls. On meta analysis of the present results with three earlier published studies on *H. bilis* an odds ratio of 1.92 (95% CI 1.06-3.47) favoring helicobacter was observed. Heterogeneity among these studies was relatively low (chi square 8.69, p=0.03). 73/124 cases and 38/87 controls were positive for *Helicobacter bilis*

(Figure 3).

Discussion

H. bilis is a gram negative, nonsporulating, microaerophilic, fusiform to slightly spiral bacteria measuring 0.5 by 4 to 5 mm with 3 to 14 multiple bipolar-sheathed flagella and periplasmic fibers wrapped around the cell. The bacterium has been isolated from the hepatic, extrahepatic and intrahepatic bile and tissue. The bacteria have been found to colonize distal small intestine, caecum and large intestine, the hepatic and extrahepatic infection has been proposed to occur through entrohepatic route. *H. bilis* is not found in stomach and hence has also been called as nongastric *Helicobacter*. The virulence factors of *H. bilis* have been divided in three broad categories depending on their functions i.e. colonization factors, persistence factors and disease inducing factors.

Culture of *H. bilis* from bile juice or biliary or biliary tract tissue is the best way to prove its presence in biliary diseases. However, more emphasis is now being placed on molecular detection of bacteria using most conserved 16S rRNA PCR. *H. bilis* was first identified in inbred mice with chronic hepatitis by using sequencing of PCR amplified

16s rRNA gene fragment analysis (Ananieva et al., 2002). It is a Gram-negative, opportunistic, fusiform, flagellated, non-sporulating, microaerophilic bacteria with pinpoint colony on thin spreading layer on agar. Using 16S rRNA specific PCR, we found that 32 of 54 gallbladder cancer resected samples were positive for *H. bilis*.

H. bilis has been proposed to play an important role in development of hepatobiliary and gastrointestinal diseases. *H. bilis* antibodies have been identified in sera of patients with chronic liver diseases and cirrhosis (Feng et al., 2004), while bacteria has also been demonstrated in patients with benign and malignant liver neoplasms like hepatoma and hepatocellular carcinoma. However, the infection is most frequently observed in biliary tract, where they have been identified in bile of patients with benign biliary diseases like gallstones, and primary sclerosing cholangitis, as well as in gallbladder and extra hepatic biliary tract cancers (Murata et al., 2004). *H. bilis* has also been found to be associated with development of colitis in humans and typhocolitis and gallstones in experimental models. Mono-infection with *H. bilis* or its coinfection with *H. hepaticus* and *H. rodentium* has been found to increase the incidence of cholesterol gallstones in humans (Maurer et al., 2005).

The presence of enterohepatic *Helicobacter* in the gallbladder carcinoma has been dubbed as a cofactor contributing in biliary carcinogenesis. However, the exact mechanism of entry of *Helicobacter* in humans and how it effects carcinogenesis are still not clear (Fox, 2002). It has been suggested that enterohepatic *Helicobacter* infection may be a factor in the development of cholesterol gallstone and intrahepatic cholelithiasis which may further lead to carcinogenesis (Maurer et al., 2005; 2007). Promotion of the risk of stone formation could be due to *Helicobacter* acting as a foreign body nidus around which the stone may develop or it may produce hydrolyzing enzymes or nucleating proteins like immunoglobulins (Pandey and Shukla, 2009). Increase in lithogenicity by production of soluble antigens that may bind to and inhibit key hepatobiliary genes like muc, and modulation of enterohepatic cycling of conjugated bile acids through genetic regulation are also proposed (Myung et al., 2000). *Helicobacter* has also been found to promote epithelial proliferation and intestinal metaplasia of the gallbladder (Arnaout et al., 1990).

Other proposed mechanisms of *Helicobacter* induced carcinogenesis include presence of virulence factor Cag A which interferes with signal transduction pathway (Maurer et al., 2005), and host response to *Helicobacter* antigens in form of cytokines, and other inflammatory mediators (Myung et al., 2000). However, based on evidence available so far, inflammation followed by epithelial proliferation and interference with cell cycle through alterations of signal transduction appears to be most plausible explanation of carcinogenesis.

The prevalence of *H. bilis* and other species of *Helicobacter* have been found in the population with low risk of non-viral hepatitis and biliary duct malignancies (Rocha et al., 2005; Matsumoto et al., 2007; Veijola et al., 2007). Two studies from India and China have shown presence of *Helicobacter* in gallbladder epithelium of

nearly 45% of the patients with gallstones especially in the areas of gastric metaplasia (Misr et al., 2007). Only three previous studies have looked at prevalence of *H. bilis* in hepatobiliary tract cancers, totaling 70 cases of which *H. bilis* was identified in 41 patients, a prevalence of 58.5%. The rate of prevalence in the present study was 59.2% which is similar to earlier observed. However, the prevalence in the gallstone controls were higher in the present study (58.2%) compared to previous reported prevalence of 18%. The present study showed no significant increase in risk of development of gallbladder carcinoma in presence of *Helicobacter bilis* infection of the gallbladder. This could be due to absence of healthy controls in the present study, and possible association of *H. bilis* with gallstone disease as earlier suggested (Myung et al., 2000; Maurer et al., 2005; 2007; Pandey, 2007).

On pooling the results of the present study to the earlier meta analysis, a lowering of odds was seen from earlier 8.72 to 4.13, which is still higher and favors a role of *Helicobacter* in hepatobiliary carcinogenesis. However, when only the studies that looked at *H. bilis* were taken (Fox et al., 1998; Matsukura et al., 2002; Murata et al., 2004) only a slight increase in odds to 1.92 was observed. These results still suggest a possible role of *H. bilis*, however, smaller sample size and high heterogeneity are two compounding factors that need to be taken into account before interpretation of these results.

Meta analysis of studies of *Helicobacter* species in gallbladder cancer (Fox et al., 1998; Murata et al., 2004; Bohr et al., 2007) failed to show any significant increase in odds (OR 1.24; 95% CI 0.63-2.44), suggesting that *H. bilis* does not have any role to play in gallbladder carcinogenesis. However, most studies have taken patients with gallstones as controls and this might have an influence on results as the risk of carcinogenesis could be through increase in risk of gallstones.

In conclusion, the results of present study and meta analysis show that the relationship of *Helicobacter* species in general and *Helicobacter bilis* in particular with gallbladder cancer is more complex than earlier thought. It appears that the risk is probably indirectly due to increase in the risk of gallstones, though lack of any study looking at this aspect makes interpretation of results difficult. Further studies with healthy controls are required to convincingly demonstrate a role of *Helicobacter* in gallstone disease or gallbladder carcinogenesis.

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