

## Contribution of Hepatitis C Virus and Helicobacter Pylori Co-Infection as Possible Predisposing Factors in the Occurrence of Gastric Mucosal Dysplasia

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### ABSTRACT

**Background and Purpose:** Current views on B-cell lymphoma genesis suggest that several exogenous factors, acting in a multistep fashion upon a predisposing condition, may be involved in B-cell clonal expansion, a potentially prelymphomatous stage. This study was done to investigate the extrahepatic localization of Hepatitis C virus (HCV) in the gastric mucosa and the possibility of its involvement besides Helicobacter pylori (*H. pylori*) as possible predisposing factors that might play a role in the occurrence of gastric dysplasia or lymphoproliferation following gastritis and may end in carcinogenesis

**Patients and Methods:** A well characterized series of 45 patients with chronic liver disease complaining of gastric dyspepsia were subjected to Upper Gastrointestinal Endoscopy and histological examination of gastric biopsy with studying the prevalence of serologic and molecular markers of HCV and *H. pylori* in the patients' serum and their gastric tissue. HCV-RNA detection in gastric tissue was done only for those who showed gastric dysplasia.

**Results:** Histopathological examination of the gastric biopsies revealed that 20 patients (44.4%) had chronic active gastritis, 15 patients (33.4%) had chronic gastritis and 10 patients (22.2%) had gastric dysplasia with chronic gastritis. As for hepatitis C virus, 38 patients (84.4%) were reactive for serum antibodies (HCV-Abs) and 18 patients (40%) showed Polymerase Chain Reaction (PCR) positivity. Helicobacter pylori antibody reactivity was detected in 37 patients (82.2%) while PCR positivity was detected in 24 patients (53.3%) both in their serum as well as in gastric tissues. Seventeen out of twenty cases showing chronic active gastritis were serologically positive for both *H. pylori* and HCV. Patients who showed dysplasia on pathological examination (n=10) were all HCV-Abs positive ( $p$ -value = 0.32), seven patients were serum HCV-RNA positive ( $p$ -value = 0.083) and 3 of them showed HCV-RNA positivity in their gastric tissue. Nine out of the patients with gastric dysplastic changes proved positive for *H. pylori* DNA both in serum ( $p$ -value= 0.027) and tissue ( $p$ -value= 0.029).

**Conclusions:** We suggest that Hepatitis C virus may be considered, in addition to Helicobacter pylori, as another potential infectious co-factor in the occurrence of gastric mucosal dysplasia and thus might be associated in the multistep hypothesis of carcinogenesis.

**Key Words:** HCV - *H. pylori* - Gastric mucosa - Dysplasia - Oncogenesis.

### INTRODUCTION

Hepatitis C virus has been localized in several tissues besides the liver [1]. The definition of the possible extrahepatic localizations of the virus may be relevant to better address the issue of some of the extra hepatic manifestations of chronic HCV infection. As regards the gastric microenvironment, gastric lymphoma has been reported in patients with HCV infection [2] and a relationship was noticed between HCV infection and B-cell clonal expansion in chronic gastritis lesions in Sjögren's syndrome [3]. Disappearance of gastric mucosa-associated lymphoid tissue in hepatitis C virus-positive patients was detected after anti-hepatitis C virus therapy [4]. The possibility is raised that HCV infection may produce, amongst a variable host response, a proliferative response of lymphoid elements to viral antigens and in combination with other factors i.e. genetic, infectious and/or environmental, may contribute to the multistep transformation in low-grade lymphoma of MALT type [5].

*H. Pylori* has been classified by the International Agency for Research on Cancer as a group I carcinogen [6]. There are increasing evidence that this infection is associated with

both the initiation and progress of gastric carcinoma and lymphoma. The presence of *H. Pylori* in gastric mucosa was associated with an increased risk of progression of the gastritis to dysplasia or gastric cancer [7]. The sequence leading to gastric cancer can schematically reduced to *H. pylori* infection-chronic gastritis-atrophy-intestinal metaplasia - dysplasia- neoplasia [8].

Low-grade lymphoma of MALT type which represents a distinct entity within B-cell non-Hodgkin's lymphoma has been now included in the WHO modification of REAL classification within the marginal zone B-cell lymphoma. In most patients a history of autoimmune disease, such as Sjögren's syndrome and Hashimoto's thyroiditis, or *H. pylori* gastritis, has been consistently reported. In particular, a possible causal role of *H. Pylori* infection has been confirmed by clinical studies documenting a regression of such lymphomas after eradication of HP infection. In addition to a possible chronic stimulation of lymphoid cells by an infectious agent, genetic host factors are likely to contribute to the development of this unusual tumor [9].

Thus, we aimed to investigate the extrahepatic localization of HCV in the gastric mucosa of patients with chronic liver disease complaining of gastric dyspepsia and the possibility of its involvement besides *H. pylori* as possible predisposing factors that might play a role in the occurrence of dysplasia following gastritis and may thus be a contributing factor in the multistep hypothesis of carcinogenesis.

## PATIENTS AND METHODS

Forty five gastric tissues and serum samples were taken from patients with chronic liver disease (i.e impaired liver function tests for more than 6 months with sonographic evidence) who came to the Endoscopy unit in El-Kasr El-Aini Hospital, Cairo University complaining of gastric dyspepsia. They included 32 males and 13 females with an age range from 38 to 68 years and a mean age of  $48 \pm 3.3$  years. Twenty normal healthy blood donors were studied as a control group.

*Pathological examination:* The gastric tissue biopsy was divided into two parts, one was examined histologically after staining with hematoxylin and eosin and the other one was sent for molecular study.

*Serologic markers for HCV-Abs and H. pylori-IgG:*

A- HCV antibodies were detected using ortho HCV 3.0 ELISA test system with enhanced sample addition verification: the Hepatitis C virus encoded antigen (recombinant C22-3, C200 and NS5) prepared under U.S. License by Chiron Corporation.

B- *H. pylori* IgG antibodies were detected using EIAgen helicobacter pylori IgG kit manufactured by Adaltis Italia S.P.A. for qualitative and quantitative determination of IgG class antibodies to Helicobacter pylori in human.

Molecular diagnosis for serum and tissue *H. pylori* DNA, serum HCV-RNA was performed for our study groups. Tissue HCV-RNA detection was done only for those who showed gastric dysplasia being a risk group.

*HCV-RNA:*

*Nucleic acid extraction:* Nucleic acid was extracted from 100  $\mu$ l of serum by a method previously described by Boom et al., [10]. HCV-RNA amplification was done by one step reverse transcriptase-polymerase chain reaction (RT-PCR) using Qiagene kit (Germany).

*The RT-PCR:* Was performed in a 50  $\mu$ l reaction volume containing 1x buffer, 1mM MgSO<sub>4</sub>, 0.2mM dNTPs, 100ng of RB6A (5' GTG.AGG.AAC.TAC.TGT.CTT.CAC.G-3' [nt 47 to 681]), 100ng of RB6B (5'-ACT.CGC.AAG.CAC.CCT.ATC.AGG-3 [nt 292 to 312]) and 1ul of RT-PCR enzyme. The samples were incubated for 45 min at 50°C, then denatured at 95°C for 5 min and were subjected to 35 rounds of thermal cycling in a DNA thermal cycler (Eppendorf, Hamburg, Germany). A cycle consisted of denaturation for 1min at 95°C, annealing for 1min at 55°C, and extension for 2 min at 72°C. After the cycling program, the samples were incubated for 10 min at 72°C.

HCV-RNA extraction from gastric tissue was done using Gentra RNA isolation kit (Minneapolis, USA) according to the manufacture instructions. In brief 5-10 mg frozen ground tissue was added to a 1.5 ml tube containing 300  $\mu$ l cell lysis solution and 100  $\mu$ l protein-DNA precipitation solution. The tube was inverted gently 10 times and placed on ice bath for 9 min after centrifugation 13000 rpm for 3 min, the supernatant containing RNA was trans-

ferred to clean tube containing 300 ul of 100% isopropanol. The tube was inverted gently 50 times and centrifuged at 13000 rpm for 3 min. The RNA was washed two times with 70% ethanol and dissolved in 50 ul RNA hydration solution.

#### *Helicobacter pylori:*

DNA was extracted from tissues using QIA amp DNA extraction kit according to the manufacturer instructions (QIA, Canada). DNA amplification was performed according to Qunsheng et al., [11] in a total volume of 50 ul master mix containing 1X Taq DNA buffer (promega, USA) (containing: 20 mM Tris HCL pH 8, 50 mM KCL, 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol, and stabilizing agent), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTPs (sigma, USA), 100 ng of sense primer 5'GCCA-AATCATAAGTCCGCAGAA3' and antisense primer 5'TGAGACTTTCCTAGAAGCGTGT-T3', 2 units of Taq DNA polymerase and 10 ul of extracted DNA. Thirty round cycles of amplification were performed in a DNA thermal cycler (Eppendorf, Hamburg, Germany). Each cycle consists of 1 min at 95°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for extension. The final cycle included an extension step for 10 min at 72°C to ensure full extension of the product.

**Detection:** After PCR amplification, an aliquot of 10 ul of the products was electrophoresed in 2% agarose gel (Sigma, USA) containing ethidium bromide. The results were visualized by ultraviolet transilluminator and photograph simultaneously.

**Statistical methods:** Statistical Package for Social Science SPSS version 12 was used for data analysis. HCV and H. pylori positivity were summarized in the form of frequency and percentage. The association between HCV and H. pylori positivity versus pathological results was done using chi-square or Fisher exact test, whenever appropriate, differences were considered significant when *p* value <0.05

## RESULTS

The clinical and laboratory characteristics of patients included in the study are summarized in table (1). Histopathological examination of the gastric biopsies revealed that 20 patients (44.4%) had chronic active gastritis, 15 patients

(33.4%) had chronic gastritis and 10 patients (22.2%) had gastric dysplasia with chronic gastritis. Hepatitis C virus antibodies were detected in 38 patients (84.4%) and 18 patients (40%) showed HCV RNA positivity. Helicobacter pylori antibody reactivity was detected in 37 patients (82.2%) while H.pylori DNA positivity was encountered in 24 patients (53.3%) both in serum and gastric tissues. Seventeen out of twenty cases (85%) showing chronic active gastritis was serologically positive for both H. pylori and HCV.

Table (1): The clinical and laboratory characteristics of patients and control.

Parameter studied	n (%)
<b>Sex:</b>	
Patients:	
Male	32 (71.1)
Female	13 (28.9)
Control:	
Male	14 (70)
Female	6 (30)
<b>Age/years*:</b>	
Patients	48±3.3
Controls	42±3.5
<b>Serum HCV antibodies:</b>	
Patients (n=45)	38 (84.4)
Controls (n=20)	4 (20)
<b>Serum HCV-RNA:</b>	
Patients (n=45)	18 (40)
Controls (n=20)	3 (15)
<b>Tissue HCV-RNA:</b>	
Patients (n=10)	3 (30)
<b>Serum H. pylori antibodies (IgG):</b>	
Patients (n=45)	37 (82.2)
Controls (n=20)	2 (10)
<b>Serum H. pylori by PCR:</b>	
Patients (n=45)	24 (53.3)
Controls (n=20)	1 (5)
<b>Tissue H. pylori by PCR:</b>	
Patients (n=45)	24 (53.3)

\* Values are mean ± SD

All patients who showed dysplastic features on pathological examination (n=10) were reactive for HCV-Abs (*p*-value = 0.32), seven patients were serum HCV-RNA positive (*p*-value= 0.083) and three (30%) showed HCV-RNA positivity in tissue molecular studies. Nine of the patients with dysplasia were positive for H.

pylori serum DNA and H. pylori tissue DNA with *p*-values of 0.027 and 0.029; respectively

Comparison between cases with dysplastic features in their pathological examination versus those without gastric tissue dysplasia as regards HCV and H. pylori status is illustrated in table (2).

Table (2): HCV & H. pylori positivity among patients with and without dysplasia.

Item	Dysplasia positive		Dysplasia negative		<i>p</i> value
	No.	%	No.	%	
<i>H. pylori</i> Abs:					
Positive	9	90	28	80	0.661
Negative	1	10	7	20	
<i>H. pylori</i> PCR serum:					
Positive	9	90	16	45.7	0.027*
Negative	1	10	19	54.3	
<i>H. pylori</i> PCR tissue:					
Positive	9	90	17	48.6	0.029*
Negative	1	10	18	51.4	
<i>HCV</i> Abs:					
Positive	10	100	28	80	0.320
Negative	0	0	7	20	
<i>HCV</i> RNA serum:					
Positive	7	70	13	37.1	0.083
Negative	3	30	22	62.9	

\**p*-values < 0.05 are considered significant

Percentage of HCV-Abs reactivity among controls was 20% (4/20) while percentage of HCV-RNA positivity was 15% (3/20). Helicobacter pylori-Abs reactivity among controls was 10% (2/20) while H. pylori DNA positivity was 5% (1/20).

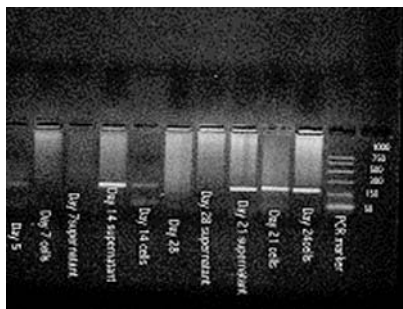


Fig. (1): PCR product of H. pylori-DNA on ethidium bromide gel electrophoresis showing positive (Lanes 1, 4, 5, 8; 9 and 10) and negative cases (lanes 2, 3, 6 and 7) and lane 11 is showing molecular size marker. Positive signals are 220 base pairs.

1 2 3 4 5 6 7 8 9 10 11 12 13

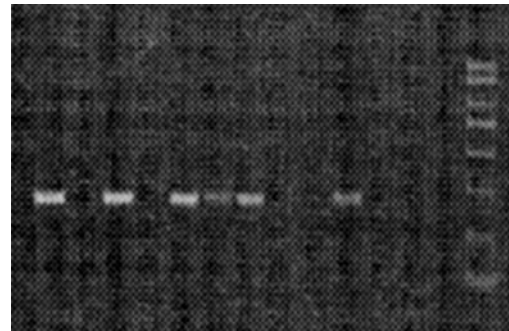


Fig. (2): PCR product of HCV-RNA on ethidium bromide gel electrophoresis showing positive (Lanes 1, 3,5,6,7 and 10) and negative cases (lanes 2, 4, 8,9,11 and 12) and lane 13 is showing molecular size marker. Positive signals are 265 base pairs.

### DISCUSSION

A major pathogenetic role of hepatitis C virus (HCV) infection in the occurrence of mixed cryoglobulinaemia (MC) type II is now well documented [5]. Recently anti-HCV antibodies and viral sequences have been observed in a substantial number of patients with non-Hodgkin's lymphoma not associated with cryoglobulinaemia, extending the spectrum of lymphoid disorders possibly related to HCV. The possibility is now raised that HCV infection may produce amongst a variable host response, a proliferative response of lymphoid elements to viral antigens and in combination with other factors; genetic, infectious and/or environmental, may contribute to the multistep transformation in low grade lymphoma of MALT type [12].

We detected 84.4% (38/45) reactivity for HCV-Abs in our patients complaining of gastric dyspepsia of which 40% (18/45) showed positivity for HCV-RNA in their serum. Ten of our patients (33.4%) showed dysplasia on histopathological examination of their gastric biopsies. We considered this group as the risk group of our patients which needs more attention in our study as well as further studies. All of them were reactive for HCV-Abs (*p* value = 0.32), 7 were serum HCV-RNA positive (*p*-value = 0.083) and 3 showed gastric tissue HCV-RNA positivity.

Although gastric dysplasia was not statistically significantly associated with serum HCV-Abs reactivity or serum HCV-RNA positivity still the presence of HCV-RNA in gastric tissue of patients with Pathological evidence of gastric

dysplasia suggests a role for HCV in the pre – neoplastic lesions that occur as a result of chronic gastritis. The duration of acquisition of HCV infection prior to the occurrence of dysplasia could not be concluded from the results of the present study but it is clear that HCV infection occurred at a previous step in the sequence of events as the majority of patients with chronic gastritis without dysplasia showed reactivity to HCV antibodies in their serum samples (28/35).

No reports are available about the prevalence of gastric dysplasia in HCV positive patients, however some authors detected HCV-localization in stomach tissue in 24/36 of HCV patients with *H. pylori* co-infection and in 6/24 HCV patients without *H. pylori* co-infection [2], other investigators reported 29.6% (8/27) HCV positivity among their patients who had Sjögren's syndrome predisposing them to B-cell clonal expansion and lymphoma. The B-cell clonality was proved by VDJ-PCR among those patients [3].

Gastric lymphomas represent approximately 5% of all gastric malignancies and are frequently due to mucosa associated lymphoid tissue (MALT). *Helicobacter pylori* induced chronic gastritis through genetic mutation of trisomy 3 and 18 leads to the development of MALT lymphoma [13,14]. The lymphocytes within this MALT are programmed to respond to the organism and the neoplastic cells of the lymphoma that may develop within this acquired MALT retain the ability to respond to the immunological proliferative drive associated with the continued presence of the organism. Following the removal of this immunological drive by eradication of the organism *in vivo*, the lymphoma shows clinical and histological regression. The time required seeing this response is unknown and there are some lymphomas that fail to respond to simple *Helicobacter* eradication [9].

In our study, we did not find any lymphoproliferative gastric changes, however Seventeen out of twenty cases (85%) showing chronic active gastritis were serologically positive for both *H. pylori* and HCV which could be a stage in the multistep hypothesis of carcinogenesis. In addition, ten out of 45 patients complaining of gastric dyspepsia proved to have dysplasia by pathological examination of their gastric

biopsies, of which 9 showed serum ( $p$ -value = 0.027) as well as tissue ( $p$ -value = 0.029) *H. pylori* PCR positivity. Thus, gastric dysplasia was statistically significantly associated with both serum *H. pylori* PCR positivity and tissue *H. pylori* PCR positivity.

These findings are supported by other authors who mentioned that the presence of *H. pylori* in gastric mucosa was associated with an increased risk of progression of the gastritis to dysplasia or gastric cancer [7]. The postulated sequence leading to gastric cancer can be schematically reduced to *H. pylori* infection, chronic gastritis, atrophy, intestinal metaplasia, dysplasia and neoplasia [8].

HCV and *H. pylori* were postulated to be involved in the pathogenesis of B-cell NHL [15]. Yet both agents were not linked to a known translocation or definite oncogenes. The exact mechanism of neoplastic transformation of both agents is still unknown. Since oncogenesis is a multistep process, possibly the persistence of infectious agents in the immune system with the consequent stimulation of clonal expansion of lymphocytes may play an indirect oncogenetic role in B-cell lymphomagenesis as an exogenous trigger rather than a direct oncogenetic role [16]. Thus, both HCV and *H. pylori* localization in the gastric mucosa may act synergistically as precancerous factors as their presence was significantly associated with moderate or marked inflammatory infiltrates and oligo-clonal heavy chain gene rearrangements [2,4,17].

We suggest that HCV may be considered an important co-factor in early stages of gastric lymph proliferation or gastric dysplasia and consequently carcinoma especially in the presence of *H. pylori* as another potential infectious co-factor in combination with other factors i.e. genetic and/or environmental. We recommend follow-up of patients with gastric dysplasia especially if combined with HCV and or *H. pylori* infection. Further studies on a large cohort of patients will be necessary to confirm our findings.

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