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A Study of the Interaction between Hepatitis C Virus Infection and Pulmonary Disorders: Assessment of Interferon Gamma and Alpha-1-Antitrypsin

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Key words: lung disease; HCV; IFN gamma; alpha-1-antitrypsin; genotype.

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Abstract

Objectives: To study lung diseases in chronic HCV infection and vice versa and to find immunological and/or hereditary interrelating factors.

Material and Methods: Study included 134 individuals, all were subjected to screening for anti-HCV antibodies by ELISA, HCV-RNA by real time PCR, pulmonary function tests, quantitative assay of interferon gamma and alpha-1- antitrypsin in serum by ELISA and genotyping of alpha-1-antitrypsin gene by Light Cycler PCR.

Results: 76.5% of chronic HCV patients had abnormal PFT (P = 0.03), mainly reduced DLCO and reversible airway obstruction, and 41.6% of chest diseased patients were HCV-positive with a significant decrease in large and small airways functions. Interferon therapy improved PFT parameters. Only 33% of chronic HCV infection affected by chest abnormality responded to interferon therapy while 50% of chest free patients did. Serum IFN-γ was higher in HCV and chest patients than in control (P=0.02). All serum α1AT deficient patients had M/null genotype.

Conclusions: A pathogenic role of chronic HCV infection in lung diseases is evident. Interferon treatment may reduce chest complications and improve pulmonary functions. However, chest affection may reduce the response to interferon treatment. M/null genotype of α1AT gene might play a role in chronic HCV infection and chest co-affection.

Introduction

An increasing number of reports have suggested that chronic hepatitis C virus (HCV) infection is associated with pulmonary disorders. The effects of HCV on the lung may present as worsening of lung function and impaired response to therapy in patients with chronic obstructive pulmonary disease (COPD) and asthma. Moreover, chronic HCV infection may be associated with the pathogenesis of interstitial lung disease [1]. Based on these reports, Kanazawa et al [2] have hypothesized that chronic HCV infection might trigger inflammation in the lungs, hence, either initiating or exacerbating the development of COPD. However, data on the prevalence of HCV infection among patients with COPD and vice versa is scanty [3]. Studies have described a high frequency (close to 30%) of anti-HCVantibodies in patients with idiopathic lung fibrosis (ILF). In some cases, pulmonary interstitial involvement may be without evident respiratory symptoms [4].

Secondary effects of HCV infection on pulmonary disease are either related to liver cirrhosis and portal hypertension or to the autoimmune disorders that are occasionally seen in association with virus infection. It is well established that chronic liver disease can lead to pulmonary derangements and mild hypoxemia is a frequent finding in approximately one third of patients [5]. The most common pulmonary problems occur due to impaired clearance of secretions and atelectasis that are associated with pleural effusions, ascites, and pulmonary edema [6]. Approximately, 10% of patients with chronic liver disease acquire unilateral or bilateral pleural effusions or "hepatic hydrothorax". In addition,

two clinically distinct syndromes that represent a continuum of pulmonary vasculopathy have been defined in association with liver cirrhosis: hepatopulmonary syndrome (HPS), representing extreme vasodilatation, and portopulmonary hypertension (PPHTN) representing vasoconstriction [7].

Interferon-alpha (IFN-α) is the current treatment for chronic HCV infection. However, interstitial pneumonitis, bronchiolitis obliterans with organizing pneumonia (BOOP), Acute respiratory distress syndrome (ARDS), pulmonary hypertension, exacerbation of asthma, and sarcoid-like disease have been described as complications in HCV patients receiving IFN [8].

One candidate for a role in pulmonary inflammation in HCV patients may be the T lymphocyte, in particular the CD8+T cell. During viral infections, cytotoxic CD8+T lymphocytes are up-regulated and activate a cascade of inflammatory pathways leading to the release of inflammatory mediators [9]. CD8+ cells are also believed to play a key role in the development of airway inflammation associated with COPD and severe or persistent asthma [10].

One of the most worldwide hereditary diseases relating liver and lung affection is the Alpha-1-antitrypsin (α1AT) deficiency [11]. The unique susceptibility of α1AT deficient individuals for exposure to chemical and particulate environmental agents can result in, both, lung and liver diseases as well as other adverse health effects [12,13]. Lung manifestations of α1AT deficiency include emphysema, chronic bronchitis, COPD, bronchiectasis and asthma [14, 15]. While liver manifestations include hepatitis, cirrhosis, hepatocarcinoma and liver failure [16].

This work is aiming to study lung disease in patients with chronic HCV infection and vice versa with a particular attention to response and/or complications to medical therapy and to find an immunological and/or hereditary factor interrelating lung and liver diseases.

Subjects and Methods

Our study included 134 adult subjects, 36 patients with chronic pulmonary diseases (bronchial asthma, COPD, bronchiectasis and interstitial lung disease), 64 patients with chronic HCV infection (positive for both anti-HCV antibodies and HCV-RNA) as well as 34 apparently healthy subjects as a control group.

All subjects were compared for risk factors for acquisition of HCV infection (blood or blood-products transfusion, major surgery, hemodialysis, occupational exposure to blood or body fluids, sexual behavior, intravenous drug abuse, dental-care procedures, shaving at barber shop, tattooing, body piercing, professional pedicure/manicure). Exclusion criteria included liver cell failure, ascites, pleural effusion and working in industrial areas. All participants gave written informed consent and the study was approved by the ethical committee of the National Research Centre.

All participants were subjected to medical history taking, clinical examination, plain chest X-ray (postero-anterior view), pre- and post-bronchodilator spirometric measurements of FEV1 **(**forced expiratory volume in one second), FEV1/FVC (forced vital capacity) and forced expiratory flow (FEF) 25/75% of predicted. Bronchodilators were stopped 24 hours before pulmonary function tests (PFT). Diffusing capacity for Carbon monoxide (DLCO), lung volumes (total lung capacity TLC and residual volume RV) were assayed in all patients with chronic HCV infection (n=64) and in chest disease patients with restrictive spirometric function (N=11).

Laboratory measurements

Screening for anti-HCV antibodies in serum was done by Enzyme linked immunosorbent assay (ELISA) using DIAKEY, ShinJin Medics Inc. Cat no. E031002. HCV-RNA was qualitatively assayed in plasma by real time PCR in patients who were positive for anti-HCV antibodies. Serum concentrations of α1AT and IFN-γ were quantitated by ELISA; kits were supplied by Immundiagnostik AG and IDlabs Inc Biotechnology, respectively. The normal range for IFN-γ is 82-102 pg/ml and for α1AT is >90 mg/dl. For patients demonstrating α1AT deficiency, genotyping of α1AT gene for the two most common deficiency alleles S and Z was done using LightCycler PCR.

Detection of HCV-RNA by Real Time PCR:

Viral RNA was extracted from patients' plasma using the QIAamp Viral RNA Kit (Qiagen Hilden, Germany, Cat no.52904) according to the manufacturer's protocol. HCV RNA was detected using commercially available Toyobo RNA-direct real time PCR kit on SLAN Real Time PCR Detection System (LG Lifescience, Korea).

Genotyping of α1-AT PI S and PI* Z alleles by LightCycler PCR*

DNA extraction: Genomic DNA was extracted from peripheral blood mononuclear cells using QIAamp DNA extraction kit (Qiagen Hilden, Germany, Cat no. 51304) according to the manufacturer's protocol. The molecular genotype of α1AT gene was detected by allele specific probe hybridization on LightCycler PCR.

Genotyping: Two PCR assays, one for each

polymorphism, were performed.

The oligonucleotide sequences for amplification in the PI* S genotyping analysis were as follows: F 5′-GGTGCCTATGATGAAGCGTTTAGGC-3′ and R 5′-AGGTGTGGGCAGCTTCTTGGTCA-3′; the size of the amplified fragment was 238 bp. Hybridization was performed with two oligonucleotide probes: 5′-GCACCTGGAAAATGAAC-3′, labelled at the 5′ end with a LightCycler red fluorophore LCR 640 (designed to hybridize over the mutation position) and 5′-TTCTTCCTGCCTGATGAGGGGAAACTA-3′, labelled with fluorescein at the 3′ end.

The oligonucleotide sequences for amplification in the PI* Z genotyping analysis were F 5′-GGTGTCCACGTGAGCCTTGC-3′ and R 5′-AAAAACATGGCCCCAGCAGCT-3′; the size of the amplified fragment was 136 bp. Hybridization was performed with the 5′-GACCATCGACGAGAAAGGG-3′ probe, labelled at the 5′ end with a LightCycler red fluorophore LCR 640 (designed to hybridize over the mutation position) and the 5′-CTCCAGGCCGTGCATAAGGCTGT-3′ probe labelled with fluorescein at the 3′ end.

The PCR conditions were identical for both applications: 3 mM of $MgCl_2$, 4 pmol of each hybridization probe, 10 pmol of the two PCR primers, 2 µl of LightCycler Fast Start DNA Master Hybridization probe mix (Roche Diagnostics), and 5 µl of DNA sample, in a total volume of 20 µl. PCR cycling conditions were as follows: an initial denaturation step of 94°C for 7 minutes, followed by 55 cycles of denaturation at 95°C for 2 seconds, annealing at 53°C for 12 seconds, and extension at 72°C for 15 seconds. After the amplification, melting curves were generated by denaturation of the reaction at 94°C for 15 seconds, holding the sample at 40°C for 20 seconds, and then slowly heating the sample to 85°C [17].

Interpretation

The normal serum level of α1-AT is associated with PI MM genotype, intermediate level is associated with PI Mnull and PI MZ genotypes, low level is associated with PI SZ genotype and finally very low level is associated with PI ZZ and PI Znull alleles. The current method for molecular genotyping is using allele specific hybridization probe that detects PI*S or *Z alleles only, thus PI Znull and PI MZ individuals will both appear to have one Z and one non-Z, non-S allele in molecular genotyping. The combination of a very low α1-AT serum protein level with genotyping can suggest a PI Znull rather than a PI MZ genotype (which would be combined with intermediate α1-AT serum protein level) [18].

Results

The study included 134 subjects. They were 59 (44%) females and 75 (56%) males. Their age ranged from 24 to 71 years. According to the results of PFT, anti-HCV antibodies by ELISA and HCV-RNA by real time PCR; they were divided into 64 patients with chronic HCV infection, 36 with chronic chest diseases (27 with bronchial asthma, 6 with COPD, 1 with asthmatic bronchitis, 1 with bronchiectasis and 1 with interstitial lung disease) and 34 were control subjects. Forty-nine patients (76.5%) of chronic HCV patients had abnormal PFT and 15 patients (41.6%) of chest diseased patients were HCV-positive. Demographic and clinical data of the studied subjects are shown in Table 1.

Table 1: Demographic and clinical data of the studied subjects.

Characteristic	Control (n=34)	Patients with HCV (n=64)	Patients with Chest disease (n=36)
Sex (F/M)	24/10	16/48	19/17
Age (years) Mean ± SD	41.41 ± 10.4	48.25 ± 9.29	45.94 ± 9.34
Smoking	0	19	8
Family history of HCV	0	3	1
H/O Major surgery	0	21	11
H/O dental care procedure	15	46	18
Other diseases:			
DM	0	7	2
HTN	0	8	2
IHD	0	2	0
HBV	0	0	0
Treated Bilharziasis	0	19	3
Treatment			
-Interferon therapy	-	16	-
Incomplete		14	
complete (48 weeks)		6	
▪ *Responder*		8	
▪ *Non responder*		-	
-Bronchodilator	-	-	3
-Inhaled steroids	-	-	3
-Both	-	-	13
-Occasional oral steroids	-	-	3

F = femal; M = male; DM = diabetes mellitus; HTN = hypertension; IHD = ischemic heart disease, HBV = hepatitis B virus.

Results of PFTs in different studied groups are shown in Table 2. A highly statistical significant decrease in the mean value of FEV1 in chest disease patients compared to patients with chronic HCV or control subjects was observed (P< 0.0001), and in chronic HCV patients compared to control subjects as well (P< 0.0001). The mean value of FEF25-75% of predicted was significantly lower in chest patients compared to chronic HCV patients, P <0.05. The mean values of FVC% of predicted and SaO2% were significantly decreased in chest disease patients and chronic HCV patients compared to control group (P <0.005 and <0.01, respectively).

Different chest abnormalities reported in patients are shown in Table 3 and Figure 1. PFT revealed that 49 cases (76.5%) of chronic HCV patients had abnormal PFT (P < 0.05).

Fifteen cases (41.6%) of chest disease patients (n=36) were HCV positive and showed a significant decrease in the mean value of FEV1 and FEF25-75% of predicted compared to HCV-negative patients, P<0.0001 and P<0.05, respectively (Table 4).

Table 2: Statistical comparison of post-bronchodilators pulmonary function test parameters between the studied groups.

Parameter					ANOVA	
		N	Mean	SD	F-ratio	P-value
FEV1%	Control	34	96.3	11.56	10.86	**< 0.0001**
	Chest	36	78.6	20.62		
	HCV	64	88.6	15.03		
FVC%	Control	34	88.1	6.43	6.45	**<0.005**
	Chest	36	79.6	17.22		
	HCV	64	79.3	11.25		
FEF25-75%	Control	34	94.8	19.47	3.65	**<0.05**
	Chest	36	80.8	47.29		
	HCV	64	100.3	32.84		
TLC%	Control	0				NS
	Chest	11	82.14	16.76		
	HCV	64	86.89	17.74		
RV/TLC%	Control	0	.	.	2.57	NS
	Chest	11	42.7	10.56		
	HCV	64	37.4	10.13		
DLCO%	Control	0	.	.	1.89	NS
	Chest	11	69.8	26.49		
	HCV	64	78.3	17.32		
DLCO/VA%	Control	0	.	.	1.82	NS
	Chest	11	84.5	30.76		
	HCV	64	95.0	22.55		
SaO2%	Control	34	97.5	.93	4.99	**<0.01**
	Chest	36	96.5	1.76		
	HCV	64	97.2	1.24		

FEV1: forced expiratory volume in one second, FVC: forced vital capacity of predicted> 85%, FEF25-75%: forced expiratory flow of predicted>60%, TLC%: Total lung capacity of predicted>60%, RV: residual volume< 33%, DLCO%: Diffusion capacity for Carbon monoxide of predicted>85%, VA: alveolar volume, SaO2: oxygen saturation> 95%. *, DLCO, TLC, RV were done for all chronic HCV patients and for cases with restrictive spirometric functions (n=11) in the chest diseases group.

Twenty patients out of 134 (14.9%) showed deficient serum α1AT (<90 mg/dl), of whom, 13 cases (65%) had combined chronic HCV infection and chest insult. Distribution of α1AT deficiency was as follows: 16.7% of chest disease patients, 16% of chronic HCV patients, 13% of control subjects, and 20.3% of combined HCV infection and chest insult patients. Genotyping results showed that all had M/null genotype (M/ nonS nonZ allele).

Table 3: Interpretation of pulmonary function tests in patient groups.

Pulmonary function test	HCV N=64	Chest N=36
Normal functions	15	0
Reduced diffusing capacity	16	-
Irreversible airway obstruction and hyperinflation	3	5
Irreversible airway obstruction and reduced diffusing capacity hyperinflation	4	2
Reversible SAW/ LAW obstruction	4	23
Reversible airway obstruction and hyperinflation	2	3
Reversible airway obstruction hyperinflation and reduced diffusing capacity	8	3
Reduced lung volume (restriction)	4	2*
Reduced lung volume (restriction) and reduced diffusing capacity	8	3*
Case with reduced sao2<95%	3	5

SAW: small airway, LAW: large airway, SaO2: oxygen saturation. *5 cases with reduced lung volumes (restriction) in Chest patients group: 2 cases with combined restriction and irreversible airway obstruction and 3 cases with combined restriction and reversible airway obstruction.

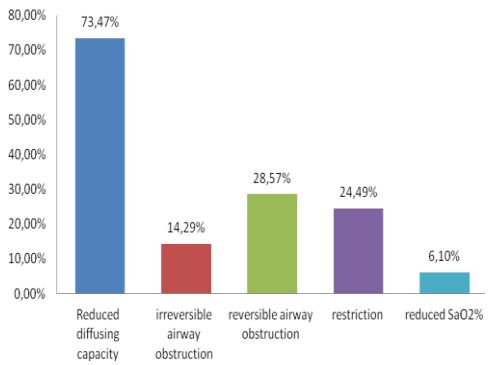

Figure 1: Different chest abnormalities in chronic HCV patients.

Table 4: Statistical comparison of pulmonary function test parameters in chest patients according to the presence or absence of HCV infection.

PFT parameter	HCV positive	HCV negative	P
	N =15	N = 21	
FEV1%	70.57 ± 19	83.63 ± 20.48	**<0.0001**
FEF25-75%	63.38 ± 38.47	92.92 ± 51.18	**<0.05**
TLC%	85.06 ± 17.47	74.33 ± 14.5	NS
DLCo %	72.86 ± 23.71	62 ± 46.94	NS
SaO2%	96.8 ± 1.78	96.26 ± 1.76	NS

Data presented as mean ± SD. FEV1%: forced expiratory volume of predicted, FEF25-75%: Forced expiratory flow of predicted, TLC%: Total lung capacity of predicted, DLCO%: Diffusion capacity for Carbon monoxide of predicted, SaO2: oxygen saturation.

Mean serum level of INFγ was significantly higher in chronic HCV patients (95.7 ± 188.5 pg/ml) than in both control group (32.6 ± 19.92 pg/ml) and chest disease patients (43.6 ± 42.77 pg/ml), P<0.05. Also, a non-statistical significant increase was found in chronic HCV- chest affected patients (96.8 ± 191.59 pg/ml) compared to control subjects (32.6 ± 19.92 pg/ml), chest disease patients (45.8 ± 51.23 pg/ml), and chronic HCV-chest free patients (46.3 ± 54.44 pg/ml), P>0.05.

Table 5: Statistical comparison of pulmonary function test parameters in HCV patients with abnormal PFT according to treatment intake.

PFT parameter	Nonspecific treatment	Interferon treatment	P
	N =33	N =30	
FEV1%	81.43±12.26	85.5±13.86	NS
FEF25-75%	84.96±28.81	104.47±29.64	**<0.05**
TLC %	81.88±20.39	85.01±17.21	NS
DLCO %	70.24±14	76.32±16.25	NS
SaO2%	97.19±0.87	97.28±1.34	NS

Data presented as mean ± SD. FEV1%: forced expiratory volume of predicted, FEF25-75%: Forced expiratory flow of predicted, TLC%: Total lung capacity of predicted, DLCO%: Diffusion capacity for Carbon monoxide of predicted, SaO2: oxygen saturation.

Studying the effect of interferon therapy on PFT, we found a statistical significant increase in the mean value of FEV25-75% of predicted in chronic HCV patients with abnormal PFT who received interferon therapy compared to patients who did not receive interferon (P <0.05). Also, mean values of FEV1%, TLC%, DLCO% and SaO2% were higher in patients who received interferon therapy compared to those who did not (Table 5). Regarding the effect of chest abnormality on the response to interferon therapy, 33% of HCV positive- chest cases responded to interferon versus 50% of chest free cases who did not.

Discussion

It is believed that chronic HCV infection may contribute to the immune responses modulating the pathogenic processes underlying pulmonary disorders and, therefore, may lead to a wide spectrum of clinical presentations [1].

We demonstrated a decline in lung functions associated with chronic HCV infection. Our results are in agreement with Okutan et al. [4] who reported a decrease of lower than 80% of the predicted value in FVC, FEV1, FEF25-75% and DLCO as well as findings of interstitial pulmonary involvement by high-

resolution computed tomography (HRCT). Similar findings were reported by Erturk et al [19] who reported that 75% of chronic HCV patients had at least one pulmonary alteration as evidenced by abnormal PFTs, implicating that pulmonary manifestations of chronic HCV are frequently under diagnosed.

Pulmonary function abnormalities in chronic liver disease are related to both the degree of hepatocellular damage and to the hemodynamic changes induced by portosystemic shunt. One of the severe complications of chronic liver disease is hepatopulmonary syndrome that is defined as a triad of liver disease and/or portal hypertension, intrapulmonary vascular dilatation and hypoxemia, in the absence of detectable primary cardiopulmonary disease [20, 21].

About 62% of patients with advanced liver disease have abnormal pulmonary diffusion capacity with a reduced DLCO or DLCO/VA. Abnormal portosystemic shunt (increased H/L ratio) is common hemodynamic abnormality. Therefore, inverse linear correlation between DLCO or DLCO/VA and H/L ratio may be an important factor in predicting pulmonary complication and meaningful diagnostic and prognostic parameters in patients with advanced chronic liver disease [22]. In our study, 41.6% of chest disease patients were HCV- positive with a significant statistical decrease in the mean value of post bronchodilator large and small airways compared to HCV- negative patients.

We demonstrated that interferon therapy improved the abnormal PFT in chronic HCV patients. Contrary to Foster et al [23] who reported frequent clinically relevant DLCO declines in chronic HCV patients receiving IFNα/ribavirin therapy that persisted for ≥6 months post therapy. Meanwhile, Kanazawa et al [24] found that interferon therapy induces improvement of lung function by inhaled corticosteroids in asthmatic patients with chronic HCV infection. Also, Kanazawa and Yoshikawa [25] reported a significantly lower reversibility with salbutamol among HCV-positive asthmatic patients who do not respond to interferon therapy than in HCV-negative patients or interferon responders. In addition, the decline in DLCO during a follow-up period of 6 years was significantly greater in interferon non-responders than in HCV-negative patients or interferon responders.

HCV activates a cascade of inflammatory pathways leading to the release of inflammatory mediators from CD8+ T lymphocytes [9]. Interferon gamma INFγ, which was taken in this study as indicator of CD8+ activation, was higher in HCV patients than in chest or control groups.

Also, IFN-γ has been shown to be a key counter-regulatory anti-fibrotic cytokine down-regulating the activity of TGF-β (stimulating and maintaining the fibrogenic process) and the extent of inhibition (or activation) of TGF-β responsive genes may be determined by the balance of TGF-β and IFN-γ signals [29], giving an interpretation of the elevated IFN-γ in our HCV patients and explaining the interstitial lung affection in such patients manifested by restrictive lung functions and reduced diffusing capacity.

Our results indicate that α1AT deficiency and M/null genotype of α1AT gene might play a role in attraction of chest diseases in chronic HCV infected patients. Our results didn't give information regarding the effect of α1AT deficiency on the response to interferon therapy as all patients who received interferon had a normal serum level of α1AT with MM genotype.

In conclusion, a pathogenic role for chronic HCV infection in lung diseases is evident. Interferon therapy improves pulmonary functions, whereas, chest affection may reduce the response to therapy. M/null genotype of α1AT gene might play a role in HCV infection and chest co-affection. Further research to delineate the underlying mechanism is needed aiming to find effective therapeutic options reducing these complications.

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