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Full Length Research

Long term follow up of sustained virological responders to interferon therapy for chronic hepatitis C genotype 4: Is there a possibility of relapse?

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Administration of pegylated interferon with ribavirin improved the virological response rates. Assessment of chronic hepatitis C outcome in sustained responders requires prolonged observation and close monitoring. To estimate the possibility of relapse among sustained virological responders (SVR) to Pegylated Interferon or Convential Interferon therapy for up to three years of follow up. Also to study the characteristics of relapsers and to test the possibility of persistence of HCV RNA in peripheral blood mononuclear cells (PBMCs) or liver tissues of SVR as a risk for relapse. Two hundred patients with chronic HCV (90% genotype IV) were included in a randomized controlled clinical trial for treatment of chronic HCV with either Pegylated Interferon or Conventional Interferon α 2b both with ribavirin for 48 weeks. Eighty-three subjects were SVR. Seventy of the responders were available for follow-up at 24 weeks interval, which was carried out by clinical assessmement and ALT levels evaluation as well as HCV RNA testing in serum, PBMCs and liver tissues. Sequencing of the HCV RNA was performed in the initial stored blood samples and in those who were viral positive during the follow up period. We followed the responders for a mean follow up period of 143 weeks (range 108-174) after end of therapy. Most of the patients (84.3%) reported the disappearance of side effects developed while on treatment with significant increase in their Body Mass Index. During the follow up period elevated ALT was found in 6% (max 1.85 folds) HCV RNA was present in 10% of the tested sera, in 1.5 % of PBMCs in absence of serum viraemia, and in none of liver tissues. Paired sequencing revealed completely different genotyping for each of the patients when comparing pre-treatment and end of follow up samples. HCV re-infection rather than relapse occurred in genotype 4 Egyptian patients with SVR to interferon based combined therapies which proved to be safe on the long term.

Key words: SVR, HCV, relapse, re-infection, PEG, INF.

INTRODUCTION

To date, combination of pegylated interferon alpha (PEG-IFN) and ribavirin is the treatment of choice for chronic HCV patients (Strader et al., 2004; NIH Consensus Statement on Management of Hepatitis 2002) with an (SVR) of 42%–52% in patients with genotype 1 (Manns et al., 2001; Fried et al., 2002) and in 42-68 % in those with genotype 4 (Esmat et al., 2002; Alfaleh et al., 2004; Derbala et al., 2005; El-Zayadi et al., 2005). Effective anti-viral treatment for chronic hepatitis C prevents long-term complications like cirrhosis and hepatocellular carcinoma (Yoshida et al., 2004; Shiratori et al., 2000).

Zeuzem and co-workers reported that 12 weeks of follow-up may be sufficient for making decisions related to the management of most patients treated with standard or pegylated interferon alpha (Zeuzem et al., 2003). However, Shinido et al found that 28% of the long term responders turned HCV RNA positive during a follow up period of 4 years (Shindo et al., 1999).

Almesio and co-workers performed a systematic review of reported data on long-term follow-up of patients with persistent HCV suppression, to obtain a combined estimate of the reduced relative risk using the random

effect model. They reported that in patients achieving a sustained virological response a relapse was observed in about 13% (range 0-86%) of subjects (Almasio et al., 2003). Some authors concluded that the optimal duration of the follow-up period of the sustained responders remains unclear. Additional prospective studies are required in order to establish an appropriate follow-up protocol for sustained responders to IFN (Formann et al., 2006).

Considering the presence or even the replication of HCV in mononuclear cells, it is possible that the virus may persist in these cells after antiviral treatment (Castillo et al., 2005). Therefore, the persistence of HCV in peripheral blood mononuclear cells (PBMCs) may constitute a reservoir for viral replication (Januszkiewicz-Lewandowska et al., 2007).

Using immunohistochemestry, and in situ RT-PCR to localize the HCV virus in liver biopsy specimens from chronically infected HCV patients treated with INF-α ,proved the HCV latency within responder livers, even 1 year after therapy cessation. It was suggested that, the lymphoid population in the liver is one of the factors that mediates relapse of the disease (Castillo et al., 2006). Follow-up biopsies performed several years after the end of treatment showed histological improvement with a significant decrease in inflammatory activity and fibrosis in sustained virological responders to IFN therapy in combination with ribavirin (Marcellin et al., 1997; Sarmento-Castro et al., 2007).

The objective of our study was to estimate the possibility of relapse among SVR to Interferon based combined therapy for up to three years of follow up and to study the characteristics of reviremics, also to test the possibility of persistence of HCV RNA in PBMCs or liver tissues of SVR as a risk for reviremia.

PATIENTS AND METHODS

Study design

Two hundred patients participated in a randomized controlled trial at the National Hepatology and Tropical Medicine Research Institute in Cairo, Egypt between July 2002 and December 2003.One hundred were treated by combination therapy of PEG INF 100 µg (Peg Intron®, Schering-Plough, Inc, Kenilworth, New Jersey) once per week plus 800 or 1000 mg/day of oral ribavirin (Rebetol®, Schering-Plough) while 100 patients received INF (Intron-A®, Schering-Plough) three million units three times per week plus 800 or 1000 mg/day of oral ribavirin. Before antiviral treatment, all anti-HCV antibody positive patients, had detectable HCV-RNA by PCR, elevated serum ALT levels for at least 6 months, negative for HBs Ag and HIV and they had not been previously treated with interferon based therapy. A liver biopsy before antiviral treatment was performed in all patients.

Out of these 200 patients, 83 had SVR, of whom 70 were available and consented for follow –up on 24 weeks basis, they were 35 subjects from each of the two treatment groups.

Patients were asked to come every 6 months for: Clinical evaluation including history taking with special emphasis on persistence of side effects and exposure to risk factors of relapse or re-infection by HCV. Alanine amino transfers (ALT) were assessed. Complete blood count (CBC) was performed to verify hematological abnormalities related to therapy while the synthetic function of the liver was evaluated by testing prothrombin concentration (PC). HCV RNA by PCR was tested in serum and in PBMCs.

Needle liver biopsy (using a 16 G hepafix needle), was performed by the end of the follow up period for patients accepting the procedure (16 patients) to be tested for: HCV RNA in liver tissues and to assess the histopathological features.

HCV RNA and genotype assessment

Follow-up data was available for 20 patients for a median of 104 (79-151) weeks they came for two visits and 50 for a median of 143 (108-175) weeks they came for 3 visits, after stop of anti-viral therapy.

Qualitative serum, PBMCs and liver tissue HCV RNA was done by direct nested RT-PCR. If the test was negative, sample purification by in-house method was done. Our laboratory protocol was validated (Abdel-Hamid et al., 1997).

In-house RT-PCR

1. RNA Extraction: was performed by QIAmp Viral RNA Kit (QLAGEN, Santa Clarita, USA).
2. Reverse Transcription and Polymerase Chain Reaction (RT-PCR): The protocol for performing RT-PCR to detect HCV RNA was performed according to Abdel-Hamid et al[20]. with modifications to increase the sensitivity of the assay. Sensitivity of the assay is 50 IU/ml.
3. Restriction fragment-length polymorphism (RFLP) procedure: From the nested PCR products, the product was digested in two different tubes by both Mva I/Hinf I in one tube, and Rsa I/Hae III in the other tube. Electrophoresis was done in a 4 % Metaphore gel.

Pathological assessment

Percutaneous liver biopsy specimens with a core at least 1 to 1.5 cm in length or encompassing at least three portal areas was stained with H&E and Masson trichrome for estimating amount of type 1 collagen.

The pathologist was unaware of clinical and biochemical data. The scoring system of Ishak (Modified Histopathological Activity Index (HAI) and modified staging) was used to assess fibrosis stage and necro-inflammatory injury (Ishak et al., 1995). Steatosis was graded based on percent of hepatocytes involved: mild <33%, moderate 33-66% and severe >66% (Brunt et al., 1999). Histological improvement was defined as reduction of the inflammatory score by 2 and fibrosis score by 1 (Sarmento-Castro et al., 2007).

Table 1: Characteristics baseline features of the studied patients

	Sex (m/f)	Age Mean ±SD (range)	Genotyp (4a/non-4a)	Pretreatment Viral load Median (range)	ALT at start of follow up normal %	Median Time (wks) of follow-up
Standard IFN*	28/7	38.63±8.08 (24-55)	32/3	0.31(.006-3.240)	33/35 (94.3%)	140 (107-174)
PEG IFN*	28/7	40.20±8.31 (25-58)	30/5	0.28(0.022-5.050)	33/35 (94.3%)	143 (108-169)
Total	**56/14**	**39.41±8.17 (24-58)**	**62/8**	**0.31(0.006-5.05)**	**66/70 (94.3%)**	**142 (108-174)**

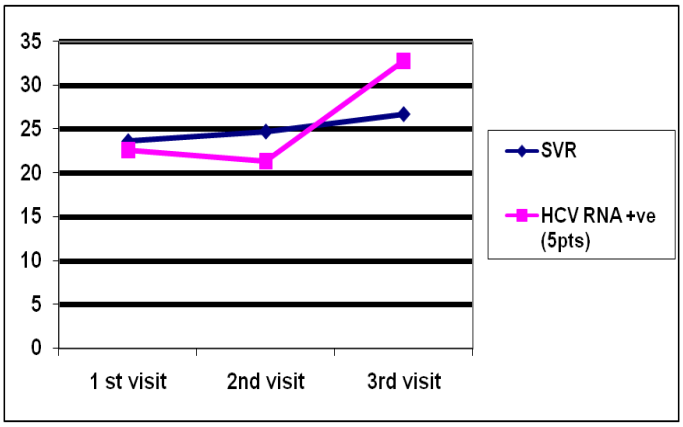

Figure 1: ALT changes during follow-up

Statistical analysis

Description of quantitative variables was by mean ± standard deviation and by median when appropriate. Qualitative variables were expressed by frequency and percentages. Paired t-student test was used to assess changes in ALT, BMI at different time intervals throughout follow up period. P value was significant al the level of 0.05.

RESULTS

Median follow-up time was 142 weeks (range 108-174, mean: 142.1). Most of the patients 59/ 70 (84.3%) reported the disappearance of side effects developed while on treatment with significant increase in their BMI 29.14±4.73 when compared with the end of treatment (26.89±3.58) ($p<0.05$) but not with pre-enrollment (28.22±4.03) BMI. PC and INR were normal in all patients. CBC showed persistent neutropenia in 7/70 (10 %) absolute neutrophilic count(1200 mm^3 -1450/mm^3) and thrombocytopenia in 3/70 (4.3%) with a range of (130x10^3 -146 x10^3/mm^3) of cases, other abnormalities of CBC were found in 33/70 and was unrelated to INF /RBV therapy ,while only 27 (38.6 %) had normal CBC .

Baseline characteristics, therapy and start follow-up data are shown in table 1. Mean age was 39.41±8.17 (24-58). Fourteen women and 56 men participated in this long term follow-up. The majority (88.57%) were infected with genotype 4a.Five of 70 (7.14%) of patients with successful anti-viral therapy had pre-treatment cirrhosis (Fibrosis grade 5-6).

ALT levels

At the start of follow up period of 70 subjects ALT level ranged 10-74 IU/ml, it was elevated in 4/70 (5.7%) with a max of 1.85 folds. By the end of follow up period ALT was elevated in 5/70 (7.1 %), ranging: 10-77 IU/ml with a max of 1.93 folds (insignificant changes) when we reviewed the ALT levels of subjects with HCV RNA positive sera a significant increase was noticed (Figure 1)

Testing of HCV RNA in Sera samples

At the start of follow up period none of our patients tested positive for serum HCV RNA. By the end of follow up period five patients relapsed (10%) 4 (80%)of them had normal ALT, all of them were genotype 4 , while the retrospective review of laboratory results showed –ve PCR at 12, 24, 48, 72 wks of the initial treatment trial as well as in PBMCs .

Testing of HCV RNA in PBMCs

One patient (1.4%) tested transiently positive for HCV RNA (once only) with no concordance to viraemia. This patient had the following characteristics: Male, obese (107 Kg), no complaints, normal ALT (18/40), Genotype 4a, PCR –ve at 12, 24, 48, 72 wks and during follow up period, was on standard IFN with no dose modification. CBC: on 2nd visit revealed leucocytosis, lymphocytosis and eosinophilia, became normal on 3rd visit.

Testing of HCV RNA in liver biopsies

Liver tissues were obtained by the end of follow –up from subjects whose sera were HCV RNA positive or negative to verify the in situ replication of HCV being the risk factor for relapse revealed that all liver biopsy tissues (16) tested –ve even those with HCV RNA positivity .

Restriction Fragment-Length Polymorphism (RFLP) Results

The five pre-treatment samples of those with HCV RNA positivity during the follow-up, had typical cleavage sites by both MvaI/HinfI and RsaI/HaeIII, they were as genotype 4a (4samples) and the fifth was 4b. End of follow up samples, one presented a genotype 3, one sample was untypable by MvaI/HinfI, and was unclassified (genotype U). Another sample had typical cleavage sites by both MvaI/HinfI and RsaI/HaeIII, this was designated as genotype 4a and 4b. A sample had typical cleavage sites by both MvaI/HinfI and RsaI/HaeIII, this was designated as genotype 4b. The last sample had

Table 2: Characteristics of patients with positive HCV RNA by the end of follow up

Patient's number	**1**	2	3	4	5
Age(yrs)/ Sex	48/F	44/M	51/M	41/M	46/M
Type of INF*	STD	STD	STD	STD	PEG
Follow-up Study duration (wks)	41.71	40.71	40.71	59.43	37.71
Pretreatment HAI (0-18)	8	7	6	5	9
Pretreatment Fibrosis Score (0-6)	2	4	3	2	2
Pretreatment genotype	4a	4a	4a	4a	4b
End of follow up genotype	3	U	4a&b	4b	4a
Duration from Clinical trial - end of study (wks)	147.14	134.29	134.29	137.86	108.57
Risk factors	Dental	unknown	unknown	unknown	unknown

*Combined with 1000mg ribavirin, no dose modification for either drug.
STD Interon
PEG peginteron

typical cleavage sites by both MvaI/HinfI and RsaI/HaeIII, this was designated as genotype 4a (Table 2).

Histopathological changes

Paired liver biopsies at baseline and at the end of follow up were available in 16 out of 50 patients who completed follow-up, 14 patients who maintained SVR and 2 re-infected patients. The inflammatory portal activity in the SVR group decreased in 9 patients increased in 3 and unchanged in 2. Similarly, fibrosis score decreased in 11 patients, increased in 2 in whom fibrosis had progressed to cirrhosis in spite of maintaining SVR and unchanged in 1. The HAI and the fibrosis score remained at the same levels in one of the re-infected subjects while the other showed HAI decrease of 3 points (from 8 to 5) and fibrosis (from 2 to 1)

DISCUSSION AND CONCLUSION

Definition of sustained response has changed within the last years. While in earlier studies, patients with normalization of ALT during treatment and in follow-up period were considered to be sustained responders (sustained biochemical response, complete/incomplete response), Strader et al., 2004, currently, SVR is defined by undetectable HCV-RNA at the end of the 6-month follow-up period. This difference in definition of sustained response limits comparison of results in earlier studies with the late relapse rates of 41% after 3 years (Saracco et al., 1993) and may explain positive HCV-RNA results years after IFN cessation in previously known as 'sustained responders' with persistent normal ALT levels.

The results of our study confirm previous observations seen in other studies that SVR is associated with a sustained elimination of HCV from serum and extend these results to patients treated with PEG-IFN/ribavirin. Following the standard IFN/ribavirin combination therapy, serum HCV-RNA remained undetectable in 92–100% of SVR with follow-up of 1–12 years (Shindo et al., 1999; Almasio et al., 2003; Schvarcz et al., 1999). In addition, liver histology improved in 94% and 93% had persistently normal ALT levels.18 Nevertheless, little is known on the outcome of patients treated with PEG-IFN/ribavirin combination therapy. Swain et al., 2004, evaluated the durability of SVR after the treatment with peg interferonalpha2a/ribavirin in 845 patients, who had participated in earlier PEG-IFN/ribavirin treatment trials and achieved an SVR. Only in seven patients (<1%), HCV-RNA was detected (after 56–154 weeks off treatment). These data indicate that the late relapse after SVR in chronic hepatitis C patients following an IFN-based anti-viral therapy is rare.

Nevertheless, there is an ongoing discussion whether the current anti-viral treatment for chronic HCV infection results in complete elimination of the virus, or whether small quantities of virus persist. In the current study none of the patients showed positive or negative strand HCV-RNA in liver tissues, even those 2 biopsies of positive sera HCV RNA patients. Previous studies have shown that 95% of the patients with SVR had undetectable liver HCV-RNA 1–2 years after therapy (Marcellin et al., 1997; McHutchison et al., 2002). Of the seven with detectable post-treatment intrahepatic HCV-RNA, only two had serological relapse up to 4 years later (McHutchison et al., 2002). Another report showed that in all 15 patients studied clearance of liver HCV-RNA was sustained up to 12 years after therapy. The absence of neither positive nor negative HCV-RNA strands in liver biopsy as well as sustained reduction in HCV core antibody titers at a constant rate further corroborated complete HCV eradication (Tsuda et al., 2004) .However, some observations contradict these results. In a small study, only two of 17 SVR to IFN/ribavirin treatment remained consequently HCV-RNA negative in all analyzed compartments, including hepatocytes, serum, peripheral blood mononuclear cells, lymphocytes and macrophage cultures. Sixty-five percent were HCV-RNA positive in macrophages, 41% in lymphocytes, and viral sequences were detected in three of 11 livers and in sera from four patients. They suggested that continuous viral presence could result in persistent humoral and cellular immunity for many years after therapy and could present a potential risk for infection reactivation (Radkowski et al.,

2005). Interestingly within this context, one patient in our study had borderline-positive PBMCs HCV-PCR results once within follow-up, but was proven to be HCV-RNA negative in control testing in serum and PBMCs soon thereafter and remained so. Whether this observation represents virus persistence with rare transient-positive PCR results is unknown.

A surprising result in our studied patients was the 10% serum HCV RNA positivity by the end of follow-up period despite its negativity at the start of study. To verify whether these cases represented a relapse of pretreatment infection or a re-infection, HCV RNA sequencing was attempted .The Restriction Fragment-Length Polymorphism Results (RFLP) of the five pre-treatment samples of those subjects and the end of follow-up samples revealed typical cleavage sites totally different for each patient confirming the possibility of re-infection. The hypothesis of mutagenesis of HCV RNA under Interferon (Puig-Basagoiti et al., 2005) and ribavirin (Asahina et al., 2005) therapy was inapplicable as these patients had negative HCV RNA at the start of the follow-up period and additionally one case showed different genotype while the 4 others showed different subtype in both samples not only quasispecies changes. The fact that only one patient had a known risk factor for re-infection (dental manipulation) and that HCV genotype 3 is rare in Egypt were verified, we found that the unapparent mode of transmission for HCV was common among Egyptians (Arthur et al., 1997; Zakaria et al., 2005). Reports of the presence of HCV genotype 3 in about 5 % of Egyptians were published (Mohamed et al., 2006; Zekri et al., 2007). An additional explanation to this high rate of re-infection is provided by the data of Mohammed et al who reported that: Over an average of 1.6 years, asymptomatic anti-HCV seroconversion occurred in 3.1/1,000 person-years, including 6.8/1,000 in a Nile Delta village, where baseline prevalence of anti HCV was 24% and 0.8/1,000 in an Upper Egypt village where the baseline prevalence was 9% (Mohamed et al., 2005).

In conclusion, no recurrence of HCV infection was seen in any patient with an SVR achieved by an IFN based anti-viral therapy. Thus, long-term prognosis in chronic hepatitis C patients with an SVR to therapy with PEG-IFN/ribavirin is excellent. Re-infection in 10 % of sustained virological responders warrants that every effort should be made to prevent community acquired infections.

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