MOLECULAR STUDY ON ENDOGENOUS INTERFERON ALPHA 2 IN HCV INFECTED PATIENTS TREATED WITH RECOMBINANT INTERFERON ALPHA

A THESIS

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By

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Abstract

Hepatitis C infection affects 170-200 million people worldwide. Egypt has one of the highest prevalence rates in the world. HCV Genotype 4 represents over 90 % of cases in Egypt. The standard therapy **for** HCV is the pegylated rhIFN-α2 plus Ribavirin. Treatment with IFN is associated occasionally with the development of anti-IFN Abs. This could be due to allelic differences between endogenous IFN-α2 and the recombinant IFN-α2 used for treatment. The aim of this research was to identify the type of IFN-α2 alleles in Egyptian cases and incidence and etiology for development of anti- IFN-α2 antibodies in Egyptian HCV infected patients treated with IFN-α, and their influence on response to treatment. The virological response was determined by using HCV nested PCR in 56 HCV infected patients had been treated with peg-IFN-α2a for 12 months. The results showed that 55.3% of those patients were responders and 44.7% were non responders and no significant difference ($p > 0.05$) had been found between the virological response and other clinical data (ALT, gender and age) of patients. Genomic DNA have been extracted from peripheral leukocytes of patients and 10 control individuals and used to isolate the genomic sequence of IFN-α2 genes using PCR. Using in vitro site directed mutagenesis technique, IFN-α2a and 2c have been constructed from the PCR product of IFN-α2b confirmed sequence and have been used as control for detection of IFN-α2 alleles. PCR method for detection of alleles using the allele specific primers did not work. Restriction analysis dependent method using *Hinf*I and *NIa*III enzymes for PCR amplified IFN-α2 genes revealed only the pattern of IFN-α2b allele for all Egyptian cases. That is suggestive for the presence of only the IFN-α2b allele or at least its predominance in the Egyptian cases. The formations of anti-IFN-α2 IgG Abs in those patients treated with different allele, rhIFN-α2a, have been assessed using ELISA. Results indicated the development of these antibodies in 18/56 (32%) of treated patients. The incidence of development of these antibodies was 6/31(19.4%) in responders, and 12/25(48%) in non-responders. The statistically significant association ($p < 0.05$) between non-responding to treatment and the incidence of these antibodies, indicates a significant influence of these antibodies on treatment outcome. No statistical significant association ($p>0.05$) had been found between the incidence of these antibodies and other clinical data (ALT level, gender and age) of patients. No significance difference had been found between reactivity of these antibodies towards rhIFN-α2a and rhIFN-α2b. So the allelic difference between the rhIFN-α2a used in treatment and endogenous IFN-α2b may not be the reason for antibody development.

Key words: HCV, HCV treatment, Endogenous IFN-α2, IFN-α2 alleles, rhIFN-α2 and Antibody response

List of abbreviations

Abs	Antibodies
Anti-IFN Abs	Anti-interferon antibodies
BAbs	Binding antibodies
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CTL	Cytolytic T lymphocyte
DAB	3, 3'-Diaminobenzidine
DEPC	diethylpyrocarbonate
DNA	Deoxynucleic acid
dNTP	Deoxy nucleotide tri-phosphate
EDTA	Ethyline diamine tetraacetate
ELISA	Enzyme linked immunosorbant assay
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HVR	Hypervariable region
IFN-α	Interferon alpha
IFN-β	Interphone beta
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISRE	Interferon stimulated response elements

KDa	Kilo Dalton
M	Mutant
NAbs	Neutralizing antibodies
NCBI	National Center for Biotechnology Information
NPIA	Non-pegylated interferon alpha
NT	Nucleotide
O.D	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAT	Parenteral antischistosomal therapy
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEG-IFN	Pegylated interferon
PMNCs	Peripheral mononuclear cells
RFLP	Restriction fragment length polymorphism
rhIFN-α2	Recombinant human interferon alpha 2
RNA	Ribonucleic acid
rpm	Round per minute
SDS	Sodium dodecyl sulfate
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response
TEMED	N, N, N`, N` –tetra-methylenediamine
UTR	Un-translated region

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INTRODUCTION

Infection with hepatitis C virus (HCV) is a global public health problem. More than 200 million people in the world are infected with HCV. Egypt has high prevalence of HCV. The Egyptian Ministry of Health (MOHP) estimated national prevalence rate of 12% (Eassa, 2007). Chronic HCV is the main cause of liver cirrhosis and liver cancer in Egypt (Roulot, 2007).

Hepatitis C virus is an RNA virus related to the flaviviridae family. RNA viruses are genetically less stable than DNA viruses and are prone to mutation during replication. Six major HCV genotypes and numerous subtypes have been identified (Raymond and George, 2002). Genotype 4 represents over 90% of cases in Egypt (Saber *et al*., 1997).

Interferons (IFNs) are antiviral proteins made naturally by some types of white blood cells. They inhibit replication of viruses. There are three classes of IFN: IFN alpha (α), IFN beta (β) and IFN gamma (γ). IFN-α is produced by peripheral blood leukocytes or lymphoblastoid cells. At least 23 different subtypes of IFN-α are known. They are 156-166 amino acid lengths proteins (Hosoi *et al*., 1992).

Interferon-α2 is 165 amino acids molecule, unlike all other IFN-α subtypes that are made of 166 amino acids. Three alleles of IFN-α2 are known and have been designated IFN-α2a, IFN-α2b and IFN-α2c, which are different by only one or two amino acids in their entire sequence (Witt *et al*., 1996).

Recombinant human IFN-α2 (rhIFN-α2) has been widely used to treat chronic HCV infection. IFN-α2 is usually administered by subcutaneous or intramuscular injection. The terminal half-life of IFN-α is 4-5 hours. Renal excretion is the predominant route of elimination. Pegylated IFN-α (PEG-IFN-α) proved to be more potent in HCV treatment (Daan and Robert, 2002).

The virological response to IFN treatment differs from patient to another. Some patients have end-of-treatment response (ETR) which refers to the absence of viremia at completion of therapy; i.e., the serum HCV RNA value is below the level of detection in their serum. This response may be sustained virologic response (SVR) with persistent absence of serum HCV RNA for 6 months or longer after therapy or followed by relapse with recurrence of detectable serum HCV RNA within 6 months post-therapy. Some patients are non responders which is defined as a failure of IFN therapy to eliminate HCV RNA from the serum during therapy (Shiffman, 2004)

Several factors influencing the virological response to treatment, one of these factors may be the emergence of anti-IFN antibodies (anti-IFN Abs). Development of anti-IFN Abs during the treatment period may cause no response or relapse in HCV patients. Humoral response against the rhIFN-α2 may be due to acquired immunogenicity during production or allelic differences between endogenous IFN-α2 and the rhIFN-α2 used for treatment, where the three genes of IFN-α2 alleles may not be possessed by each recipient, so that antibody against a certain IFN-α2 allele would develop in recipient who does not have the corresponding IFN-α2 gene (Leroy *et al*., 1998).

It has been found that, there are distinct geographical distributions of the three IFN-α2 genes (Hosoi *et al*., 1992). The use of rhIFN-α2 allele the same like the IFN-α2.genes possessed by the recipient may help to avoid antibody responses and increase the response to treatment

AIM OF WORK

In this work we aimed to 1) identify the type of IFN alleles in Egyptian cases, 2) estimate the incidence and etiology for development of anti-IFN-α2 Abs in Egyptian HCV infected patients treated with IFN-α and 3) the influence of these antibodies on response to treatment. This will be done through the molecular study of IFN-α2 alleles in Egyptian patients treated with IFN-α2a and determination of the incidence of anti-IFN-α2 Abs in those patients and correlate such findings to response for treatment.

LITERATURE REVIEW

1) Prevalence of HCV in Egypt

Egypt has one of the world's highest prevalences of HCV infection (Darwish et al., 2001), The most remarkable feature of HCV is its ability to establish chronic infection, which occurs in 55-85 % of patients (Liang *et al*., 2000). Chronic infection is the main cause of liver cirrhosis and liver cancer in Egypt, and indeed, one of the leading causes of death. In Egypt, the major route of exposure appears to be due to medical therapy and inadequate sterilization techniques and supplies. In addition to blood transfusions, the major risk factor associated with HCV infection is a history of antischistosomal injection treatment. Schistosomiasis is a common parasitic disease in Egypt acquired through swimming or wading in contaminated irrigation channels or standing water. Thus, farmers and rural populations are at greatest risk, and this is supported by the higher prevalence rate of HCV in the Nile delta and rural areas (Zainab *et al*., 2006).

The prevalence of HCV in Egypt may be from the use of unsterile injection equipment during mass treatment of the general population with parenteral antischistosomal therapy (PAT) (Frank *et al*., 2000). PAT was extensively practiced in Egypt from the 1920s to the 1980s and was gradually replaced by oral treatment from the 1970s onward. The most common PAT drug, tartar emetic (potassium antimony tartarate), was administered over the course of a few weeks as a series of intravenous injections.The potential for transmission of blood-born pathogens in such circumstances is considerable. Cross-sectional epidemiological analyses have provided evidence for the PAT hypothesis; there is a correlation between the level of exposure to PAT and HCV prevalence among different age groups and geographic regions (Pybus and Drummond, 2003).

2) HCV genotypes and replication cycles

2.1) Viral transmission

HCV is transmitted chiefly through direct blood-to-blood contact. Many cases of HCV were acquired from blood transfusions received in the early 1990s, prior to the development of reliable screening methods. Currently, the risk of being infected with HCV due to a blood transfusion is only about 0.001% per unit transfused. However, patients transfused prior to 1992 are at greater risk of developing chronic HCV (Strickland *et al*., 2005). Patients transfused during this period who are unaware that they had a transfusion (e.g., women transfused during a Cezerian operation or critically ill neonates) (Marine-Barjoan et al., 2007).

HCV may also be contracted through intravenous drug use, by using an HCV-contaminated needle, or by a needle stick. Collectively, percutaneous inoculation accounts for most new cases of HCV. Although uncommon, HCV may be transmitted through body fluids such as semen, saliva, urine, tears or vaginal fluids. Transmission of HCV during sexual intercourse is possible, but rare. The risk of a HCV patient transmitting the virus to his/her sexual partner is 5% over 10–20 years. The risk of transmission during intercourse is greater when an infected female is menstruating. While HCV is not considered a sexually transmitted disease, it does appear that individuals with multiple sexual partners are at greater risk of having HCV. About 5% of infected mothers transmit HCV to their children in utero. There is no evidence to suggest that HCV is transmitted during lactation (Major et al., 2002)

2.2) Viral genotypes and quasispecies

The term genotype refers to different genetic variations or strains of HCV. The variance in genetic differences is approximately 1/3 between the different genotypes. There are six major groups or genotypes numbered 1 to 6 although some experts believe that there may be as many as 11. Within each genotype are further divisions called subtypes (for example 1a and 1b) and

the mutation of the HCV called quasispecies (Simmonds, 2004). HCV constantly changes and mutates as it replicates more than 1 trillion HCV virions replicate each day. During the replication process, the HCV will make 'bad' copies or errors in the genetic make-up of the newly replicated viruses. Scientists believe there are literally millions of different HCV quasispecies in everyone infected with HCV (Smith and WU, 2002).

The different genotypes have different geographic distributions. To characterize the HCV genotype distribution and concordance of genotype assessments on the basis of multiple genomic regions, specimens were obtained from blood donors in 15 geographically diverse governorates throughout Egypt. The 5' noncoding, core/E1, and NS5B regions were amplified by reverse transcription-polymerase chain reaction and analyzed by both restriction fragment length polymorphism (RFLP) and phylogenetic tree construction. For the 5' noncoding region, 122 (64%) of 190 specimens were amplified and analyzed by RFLP: 111 (91%) were genotype 4, 1 (1%) was genotype 1a, 1 (1%) was genotype 1b, and 9 (7%) could not be typed. Phylogenetic analyses of the core/E1 and NS5B regions confirmed the genotype 4 preponderance and revealed evidence of 3 new subtypes (Ray et al., 2000).

2.3) HCV genome organization

HCV has a similar genomic organization and polyprotein hydrophobicity profile as the pestiviruses and flaviviruses (Choo *et al*., 1991) and has been classified as a separate genus in the family flaviviridae (Francki *et al*., 1991) . The HCV viral particle is about 50 nm in diameter (Shimizu *et al*., 1996) and consists of an envelope derived from host membranes into which are inserted the virally encoded glycoproteins (E1 and E2) surrounding a nucleocapsid and a positive-sense, single-stranded RNA genome of about 9500 nucleotides . The genome contains highly conserved untranslated regions (UTRs) at both the 5' and 3' termini (He XS *et al*., 1999), which flank a single ORF encoding a polyprotein of 3,000 amino acids (Choukhi *et al*., 1998).