Induction of interferon-gamma secretion by HCV-specific epitopes and its clinical implication in patients with chronic hepatitis C

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Induction of interferon-gamma secretion by HCV-specific epitopes and its clinical implication in patients with chronic hepatitis C

Directed by Professor Kwang-Hyub Han

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This certifies that the Master's Thesis
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pipetting 한번 해본 적 없던 제가 실험을 하겠다고 찾아왔을 때 부족함과 철없음도 늘 눈감아 주시며 선생님처럼, 선배님처럼 또는 언니처럼 같이해준 장혜영 선생님, 실험을 비롯한 모든 것과, SOS 칠 때 마다 궂은일은 도맡아 해결해 주고 도와주었던 용욱오빠, 동기라는 이름만으로도 힘이 되어주며 많은 것을 대신해 주던 용광오빠, 대학원 생활의 추억을 같이 나눈 슥인언니, 그리고 이제 막 같은 배에 올라선 효정언니, 실험실 생활을 가르쳐 주어서 너무 감사했습니다.

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Induction of interferon-gamma secretion by HCV-specific epitopes and its clinical implication in patients with chronic hepatitis C

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As interferon/ribavirin-based standard therapy is effective in only about half of HCV patients, there remain important needs for alternatives, including vaccines. Several of the cytokines (IFN-γ) that regulate of immune responses and play an important role in the control of virus-associated disease, including HCV. Therefore, differences in the productivity of IFN-γ in patients with HCV infection may account for the outcome of interferon monotherapy.

Thus, we investigated HCV specific IFN-γ productivity by HCV-specific

epitopes in patients with sustained virological responders (SVR) and non responders (NR) to interferon therapy.

A total of 40 chronic hepatitis C (CHC) patients were enrolled: 21 SVR patients and 19 NR patients. Peripheral blood mononuclear cells (PBMCs) were collected from heparinized blood of 40 CHC patients by ficoll gradient centrifugation. After in vitro culture for 5 days with each HCV-specific peptide and rhIL-15, the IFN-γ productivity were measured by enzyme-γ linked immunospot (ELISPOT) analysis and flow cytometry. HLA typing and virus genotyping were performed by Micro SSP HLA class I DNA typing and Radioimmunoprecipitation (RIPA) ssay, respectively.

Most of CHC patients had various HLA supertypes (HLA-A2 (27.5%), HLA-A3 (42.5%), HLA-B7 (30%)). The median age was 49.8 years (M:F=2.4:1). Our results showed high IFN-γ productivity percentage that IFN-γ produced by CD8+ T cells against HCV-specific epitopes in some of patients with NR when compared with SVR group.

In conclusion, HCV-specific epitopes might be used as a new therapeutics to overcome suppressed cellular immune status in CHC patients with NR for interferon /ribavirin-based standard therapy

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Key words : Hepatitis C Virus (HCV), HCV-specific epitopes, IFN-γ productivity, Sustained Virological Responders (SVR), Non Responders (NR), Interferon.

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I. INTRODUCTION

Since its discovery in 1989, hepatitis C virus (HCV) has been recognised as a major public health problem. According to the World Health Organization (WHO) approximately 170 million people worldwide suffer with chronic HCV infection [1]. HCV is a positive-stranded enveloped RNA virus belonging to the family of *Flaviviridae.* Its 10 kilobase genome of approximately 9,500 nucleotides that encodes a polyprotein of approximately 3,000 amino acids. That contains a single open-reading frame giving rise to a polyprotein which is posttranslationally processed into structural (Core, E1, E2/NS1) and non-structural proteins (NS2, NS3, NS4, and NS5) [2,3]. Analysis

of the HCV genome has demonstrated extremely high heterogeneity in both structural and nonstructural coding regions and has identified at least six different genotypes that have generally been divided into several subtypes. Differences in genotypes are significant and can be as much as 40% at the nucleotide level. Besides being restricted to different geographical areas, each genotype has its own unique pattern of disease progression and response to therapy [4].

Currently there is no vaccine or small molecule therapy for this disease, which can lead to liver failure and cancer. The majority of those infected fail to clear the virus and 20% of persistently infected individuals will develop liver cirrhosis or hepatocellular carcinoma occurs in up to 2.5% [5]. Our current peginterferon (pegasys)/ribavirin combination therapies are effective in only a fraction of the patients and are plaques with adverse effects. Furthermore, the most effective treatment is pegylated interferon (IFN)-plus ribavirin, which has morbid side effects, variable cure rates, and high costs [6]. Thus, new treatment regimens are needed that are more efficacious and better tolerated by all patients.

Investigators have taken several different approaches to address this pressing medical need. Major research efforts have focused on the identification of agents that inhibit specific steps in the life cycle of the virus. The other thing that research modulate the host immune response are being investigated for their ability. Actually, There are investigational compounds [7].

Also, rapidly emerging viral diseases, such as the hepatitic C virus (HCV), oblige the development of procedures for the fast development of vaccines.

Traditional vaccine developed efficient methods for epitope identification and vaccine design. This analysis is to predict peptides capable of binding to

specific major histocompatibility complex (MHC) molecules. In humans, MHC molecules are called human leukocyte antigen (HLA) and the main anchor residues of HLA Class Ⅰ molecules occur at positions 2 and at the C-terminus of the peptides, which are usually 8 to 11 amino acid residues in length. T cell receptor (TCR) on the T cell surface recognizes the peptide presented on the self MHC molecule. TCR of CTLs recognizes only linear determinants of epitope peptides defined predominantly by primary amino acid sequences that assume extended conformation within the peptide-binding clefts of class Ⅰ molecules. Previous studies containing a limited number of peptides will not be applicable to a large part of the human population, since most T cell epitopes are specific for single HLA allele [8,9,10]. Thus, our study used of HCV-specific epitopes capable of binding alleles representing as few as three HLA alleles, HLA-A2, HLA-A3, and HLA-B7 and these synthesized base on the referances [11,12].

Primary HCV infection causes immune evasion of broad and multispecific CD4+ and CD8+ T cell responses. A variety of postulated mechanism are summarized in *David et al* [13]. HCV antigen-exposure to immune system in individuals who develop persistent infections is usually weak or absent when compared with spontaneously resolving infections [14,15,16,17].

When exogenous antigen entered into cell as peptides, it could be also presented on MHC class Ⅰ and recognized by CD8+ T cells. In particular, cytotoxic T lymphocytes (CTLs) paly an important role in the control of virus-associatied diseases. Several groups have analyzed HCV-specific CTLs responses to identify CTLs-based epitopes within viral antigens [18-23]. It has been reported that stronger, broader and more sustained Th1/ Tc1 (IFN-γ) responses are associated with resolving infection. Although chronically infected patients also show some IFN-γ responses, these tend to be weaker and directed against less epitopes. Thus, IFN-γ producing CD8+ T cells may play an important role in the control of HCV antigen and correlation between IFN-γ levels and T cell function in CHC [24].

Also, chronically infected patients identified consistent differences hepatic gene expression in patients who SVR and NR with interferon therapy [25].

Thus, we have investigated the relationship between IFN-γ productivity differences by HCV-specific epitopes and its clinical implication in patients with CHC according to the differences outcome of interferon therapy.

II. MATERIALS AND METHODS

1. Study subjects

A total of 40 Korean subjects having either present or past evidence of HCV infection were enrolled from the patients clinic of the liver unit or from Yonsei University Medical Center between May 2004 and December 2005.

Diagnosis of CHC was based on the following criteria: (1) exposure to HCV-infected blood, (2) detection of serum HCV RNA positive at least 6 months, and (3) elevation of serum alanine aminotransferase (ALT) levels at least 6-fold above the upper limit of normal value (22U/l), and absence of markers for other viral hepatitis and autoimmune hepatitis. Patients treated interferon/ribavirin-based standard therapy for 6 month were of followed-up after the end of therapy. Subjects were classified according to the response to interferon into two groups as follows, "Sustained virological responders (SVR) group" contained 21 subjects, who have HCV-RNA negative for at least 6 months followed-up after the end of therapy, "Non responders (NR) group" contained 19 subjects who didn't showed sustained virological remission during IFN treatment.

HLA typing and virus genotyping were performed by Micro SSP HLA class I DNA typing and RIPA assay, respectively.

2. Synthetic HCV-specific epitopes

HCV-specific epitopes were synthesized and purified by a solid-phase and pin-method using the Fmoc-based protocol. Synthetic peptides at 10 mg/ml were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS) to 1 mg/ml for the binding assay. The

HCV-specific peptides were obtained by Mogam Biotechnology Institute (synthesized by Peptron Inc., Daejeon, Korea), and each was dissolved in buffer according to the chemical properties of the peptides for *in vitro* stimulation of T cells. The peptides, were resuspended in distilled water and sonicated for homogenization. Each solution of peptide was adjusted to a final concentration of 1 mg/ml and neutral pH. The purity and integrity of the synthesized epitopes were more than 95% as confirmed by HPLC.

3. Detection of intracellular cytokine staining (ICS)

Epitope-specific IFN-γ productivity were measured by IFN-γ secreting cells among CD8+ T cells after in vitro culture for 5 days with each epitopes and rhIL-15 (rhuman IL-15, R&D system) by intracellular cytokine staining (ICS) analysis. Briefly, 40 mL of blood was drawn and PBMC isolated by Ficoll-Hypaque density gradient centrifugation. The cells were resuspended in RPMI 1640 plus 10% FCS plus 50u/ml penicillin, 50μg/ml streptomycin and 2mM L-glutamine (R10) to a concentration of $5x10^5$ PBMC/well (96-well plates). Separate wells were set up with $5x10^5$ PBMC/well to which was added HCV-specific epitopes at a final concentration of 20μg/ml and rhIL-15 at a concentration of 10μg/ml. At the end of the incubation, the cells were washed twice and stained for 30 min at 4℃ with fluorescence isothiocyanate (FITC)-conjugated anti-mouse CD8 in FACS buffer [1% FBS, Gibco].The permeabilized cells were fixed with cytofix/cytoperm (BD Pharmingen), and washed with Perm/Wash buffer (BD Pharmingen). Cells were stained with R-pycoerythrin (R-PE)-conjugated anti-mouse IFN-γ (BD Pharmingen) for 30 min at 4℃, and acquired with a FACSCalibur (Becton Dnckinson) and then analysed using CellQuest software (Becton Dnckinson).

Ⅲ. RESULTS

1. The clinical characteristics of subjects

In a total of 40 patients with CHC were grouped into 21 patients of SVR and 19 patients of NR according to the interferon response (Table 1). In 21 SVR patients, median age was 52.4 years (range 36-63 years). In 19 NR patients, median age of subjects was 47.1 years (range 39-62 years). There is no considerable clinical characteristics difference between of the two groups.

Table 1. The clinical characteristics of subjects

Characteristics (Number of patients)	Sustained virological responders (SVR) (N=21)	Non responders (NR) (n=19)
Gender (M:F ratio)	1.8 : 1	2.2 : 1
Age (years)	52.4 (36-63)	47.1 (39-62)
HCV genotype (1b:Non-1b ratio)	1 : 1.29	8 : 1
HLA supertype		
A2	5	6
A3	10	7
B7	6	6

2. HCV-specific epitopes - the evidence base

Synthetic peptides with the length of 9-10 amino acids derived from HCV core, non-structural NS4, NS5B regions (Table 2). These peptides have been recognized by human PBMC's using cytolytic assays, and/or ELISPOT assays/ICS assays (IFN-γ).

Table 2. Synthetic HCV-specific epitopes sequence

Supertype	Peptides	Position	Protein	Sequence	Reference
A2	C36	36-44	core	LLPPRGPRL	[11]
	C132	132-140	core	DLMGYIPLV	[26]
	NS4-1789	1789-1797	NS4	SLMAFTAAV	[27]
A3	NS5B-2510	2510-2518	NS5B	SLTPPHSAK	[28]
	NS5B-2588	2588-2596	NS5B	RVCEKMALY	[39]
B7	C41	41-49	core	GPRLGVRAT	[30]
	C110	110-118	core	DPRRRSRNL	[12]

3. Production of IFN-γ by CD8+ T cells

Antigen-experienced CD8+ T cells upregulated IFN-γ production after encountering cells displaying their cognate HCV-specific epitopes ligand in association with a MHC complex class Ⅰ molecule. The data is representation of intracellular cytokine staining (ICS) (Figure 1).

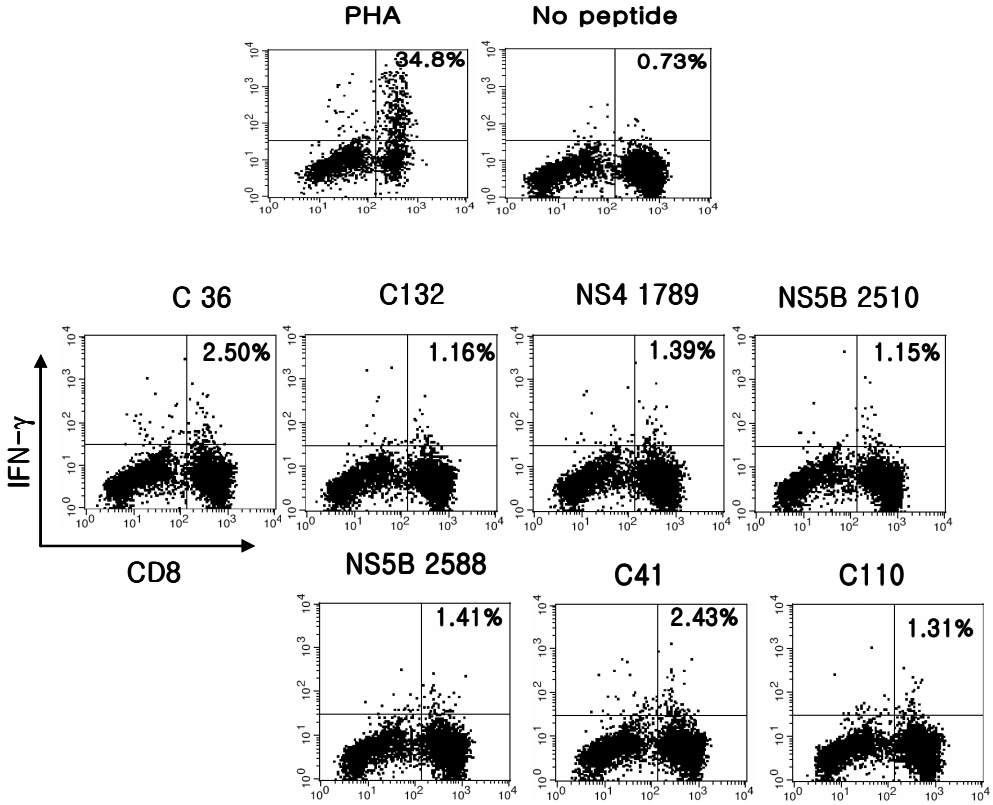
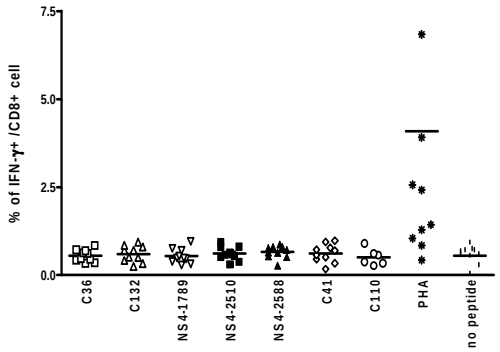


Figure 1. Detection of IFN-γ producing cells in CD8+ T cells from HCV-infected patients (PBMCs). Single-cell suspensions were prepared. Cells were stained with anti-CD8-FITC and anti-IFN-γ-PE. The percentage of $CD8^{+}$IFN-γ^{+} cells to compared with control.

4. ICS assay analysis of CHC patients and control samples to HCV-specific epitopes

A. Control samples



B. CHC patients

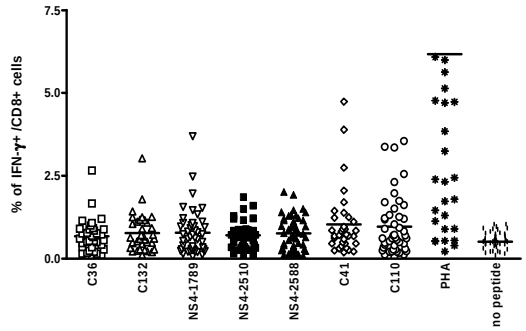
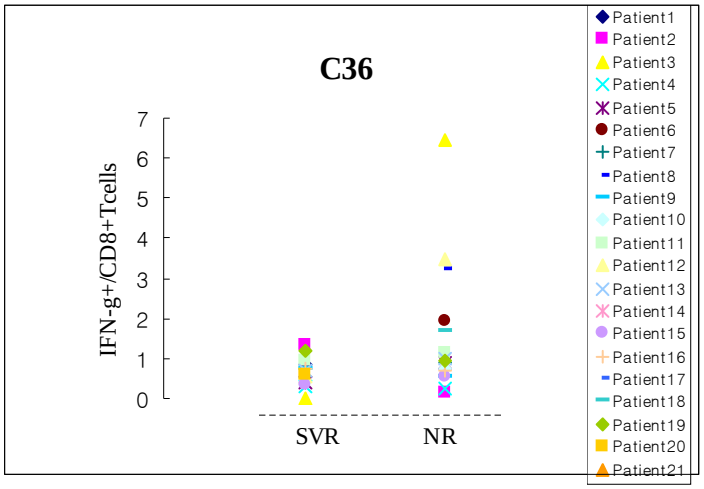


Figure 2. Epitope-specific average response by ICS assay. The IFN-γ productivity percentage of healty control responding to HCV-specific epitopes are shown (A), and CHC patients responding to HCV-specific epitopes are shown (B). A significant cut-off value percentage was more than 1% between (A) and (B). note. control samples : healthy persons.

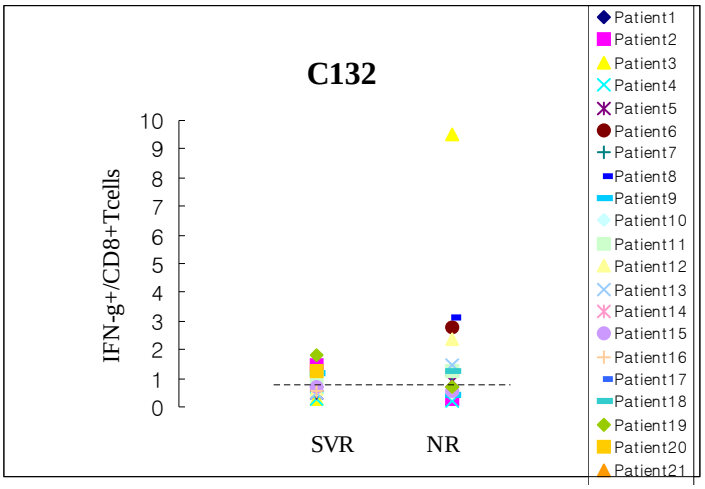
5. Frequency of HCV-specific CD8+ T cells in the peripheral blood of SVR and NR patients.

Expression of IFN-γ producing cells in PBMCs from SVR and NR patients by various HCV-specific epitopes (C36, C132, NS4 1789, NS5B 2510, NS5B 2588, C41, C110). Comparison of IFN-γ expression among SVR (n=21) and NR (n=19) (Figure 3). IFN-γ productivity was rated positive if peptide-specific IFN-γ expression was > 1%.

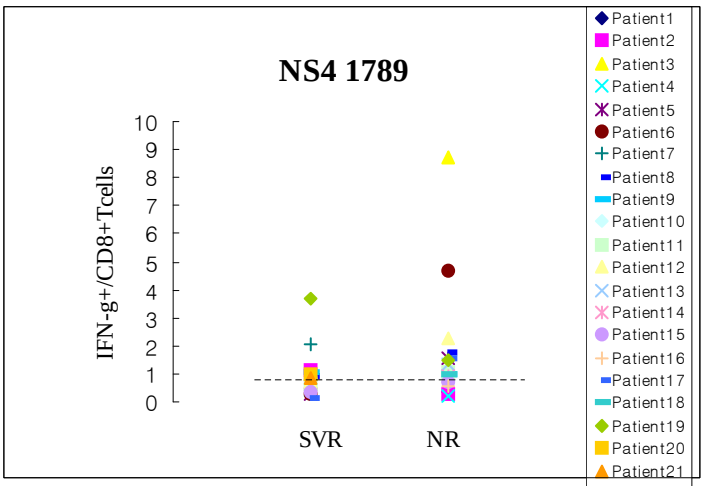
(a) HCV-specific epitope C36



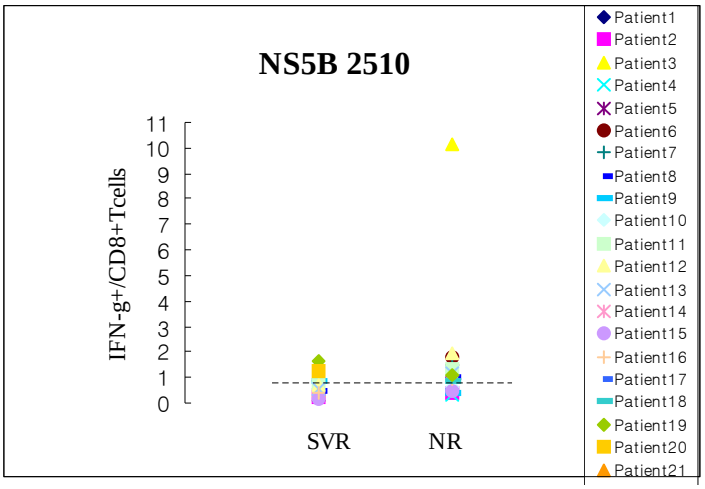
(b) HCV-specific epitope C132



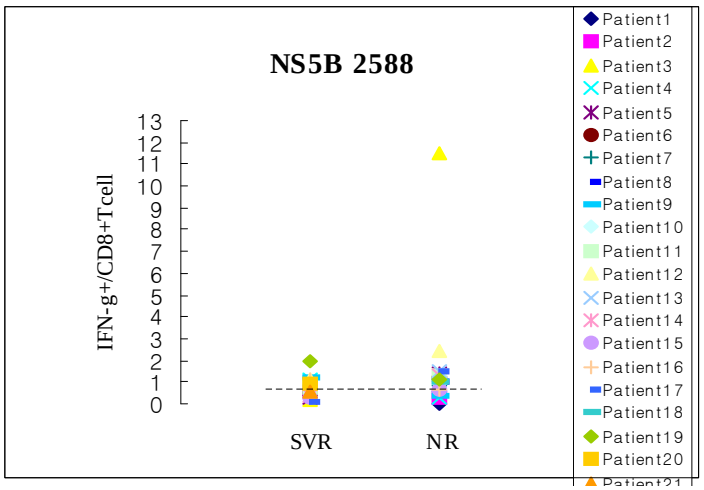
(c) HCV-specific epitope NS4 1789



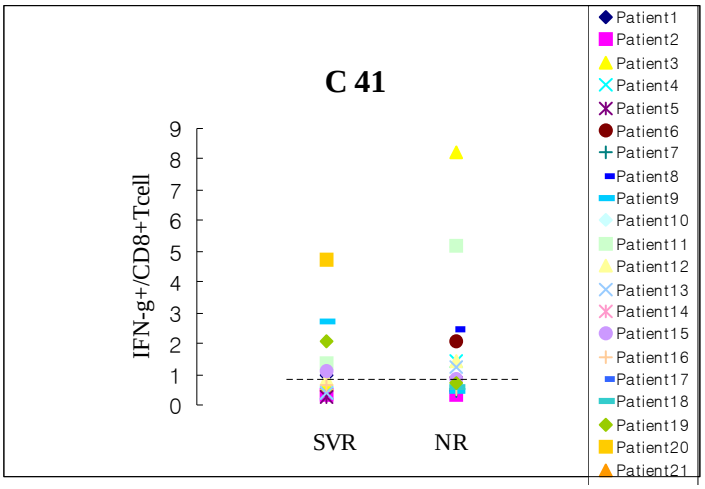
(d) HCV-specific epitope NS5B 2510



(e) HCV-specific epitope NS5B 2588



(f) HCV-specific epitope C41



(g) HCV-specific epitope C110

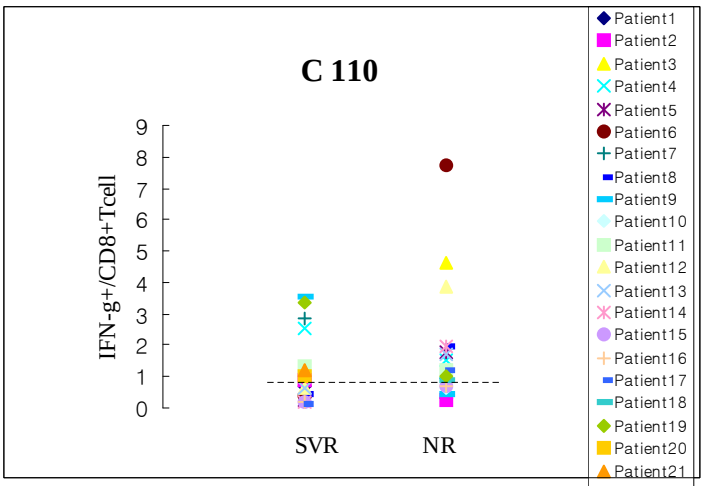


Figure 3. Comparison of IFN-γ production in response to HCV-specific epitopes (a) C36, (b) C132, (c) NS4 1789, (d) NS5B 2510, (e) NS5B 2588, (f) C41, (g) C110 among patient group (SVR *vs* NR). Each dot represents the individual SVR patients (n=21) and NR patients (n=19).

6. Pattern of CD8+ T cells responses to HCV-specific epitopes

The positive percentage of HCV-antigen-specific IFN-γ production assays is shown for patients with SVR and NR in relation to the protein location of the HCV-specific epitopes (Table 3).

Table 3. Pattern of CD8+ T cells responses to HCV-specific epitopes

	HCV-specific epitopes						
	C36	**C132**	**NS41789**	**NS5B2510**	**NS5B2588**	**C41**	**C110**
SVR							
RN/TN (%)	3/15 (20)	6/15 (40)	6/21 (28.6)	4/17 (23.5)	6/19 (31.6)	7/15 (46.7)	8/21 (38.1)
NR							
RN/TN (%)	4/15 (25)	8/16 (50)	11/19 (57.9)	7/16 (43.8)	8/19 (42.1)	6/16 (37.5)	9/19 (47.4)

Note. Percentage was calculated by the ratio of patient number showing the response to each epitope to total patients number (RN / TN).

IV. DISCUSSION

The outcome of many viral infections is determined by the nature of the antiviral immune response. However it is well known that the cellular immune responses to be too weak to eliminate HCV and a functional suppression has been reported in chronic HCV infection [31,32,33,34,35].

We identified that IFN-γ productivity to HCV-specific epitopes that distinguish patients with SVR and NR after the end of IFN therapy. IFN-γ producing CD8+ T cells was confirmed by ICS assays in figure 1 (Mogam Biotechnology Institute).

These data showed the most of patients recruited strong IFN-γ productivity after *in vitro culture* with these epitope peptides and IL-15, while freshly collected bloods from 40 CHC patients secreted little amount of IFN-γ to selected epitopes stimulation (data not shown). These results thought about antigens are provided by professional antigen presenting cell expressing co-stimulatory molecules or producing cytokines of IL-15. Actually, recent studies have shown that IL-15 can positively regulate the maturation processes as well as functions of DCs to generate efficient pathogen or tumor-specific CTLs responses [36,37,38]. IL-15 also mediates pleiotropic actions by driving the proliferation and maintenance of Ag-specific memory T lymphocytes as well as the production of TH1-diven cytokines [39,40,41].

We have found that the differences of IFN-γ productivity between in some of patients with NR and in some of patients with SVR. The IFN-γ productivity showed higher frequency in NR group than in SVR group. Also, some patients with NR have that more recognize NS4 1789 peptide than SVR , relation to the protein location of the HCV antigen (28.6% *vs* 57.8%).

Interestingly, however, these results indicated that strong IFN-γ induced

by CD8+ T cells to HLA-restricted A2, A3, B7 peptides in patients with NR. These results are consistent with recent studies showing higher frequency of IFN-γ producing peripheral CD4+ and CD8+ T cells in HCV RNA-positive patients than in HCV RNA-negative patients or healthy controls [42]. Also, *Fareed Rahman and Barbara Rehermann et al* [43] compared to patients sustained treatment response and patient with viral breakthrough. HCV-specific T cells responses showed some fluctuation with significant peaks of IFN-γ producing T cells according to ALT levels and HCV-RNA. These results suggest that HCV-specific T cells responses may have been stimulated and maintained by low levels of remaining antigen and that HCV may not have been completely cleared.

However, all of the patients didn't response to HCV-specific epitopes. One of the candidate answer of this question is a diversity host factors. For exemples, there are HCV genotype, gender, sex, ethnic, and age. Actually, specific HLA alleles have been reported to be associated with response to therapy in chronic HCV infection [44]. In addition, the role of single nucleotide polymorphisms (SNPs) in some specific genes related to disease and treatment response, in considering the host genetic factors, has become increasingly important in a variety of diseases.

It is possible that other host protein with different functions may also be associated, through different mechanisms, with the IFN responsiveness of the HCV-infected patients. The MxA protein is one of such candidats, because it is a protein that is induced by IFN and influences the IFN-induced antiviral activities of host cells against several other viruses [45,46,47]. Also, a polymorphic site identified in the promoter region of MxA was involved in a region which shows a considerable resemblance to the IFN-stimulated response element (ISRE) [48].

The traditional attempts to develop single peptide vaccines, based on a limitied number of peptides face two problems : HLA polymorphism and the escape mutant. Thus, single peptide based vaccine containing a limited

number of peptides will not be applicable to a large part of the human population, since most T cell epitopes are specific for a single HLA allele [49]. However, the use of supertype-restricted multipeptides, those capable of binding with significant affinity to multiple related HLA alleles. Thus, we used of HCV-specific multiepitopes capable of binding alleles representing as few as three HLA alleles, HLA-A2, HLA-A3, and HLA-B7, results in redicted recognition in nearly 90% of the global population, regardless of ethnicity.

Actually, we performed HLA typing assay by Micro SSP HLA class I DNA typing respectively from patients. The most of patients have a various HLA supertype (HLA-A2 (27.5%), HLA-A3 (42.5%), HLA-B7 (30%)) showed that in Table 1. But, we didn't check up association with between HLA type and IFN-γ productivity. Thus, whether HLA type might be related to the IFN-γ productivity and differences outcome of interferon therapy or not, further study may be needed.

Our experiment is detection IFN-γ producing CD8+ T cells *in vitro culture*, the IFN-γ productivity showed higher frequency in NR group than in SVR group. Taken togethers, these HCV-specific multiepitopes might contribute to overcome suppressed cellular immune status in CHC patients with NR for interferon /ribavirin-based standard therapy. It also gives the possibility that mutiepitope-based DNA vaccine vectors could be design. But, our experiment is just detection of patients with the end of treated interferon/ribavirin-based standard therapy. Therefore, further study may be needed a before starting drug treatment and during the therapy. In addition, further detailed study is needed to evaluate the regulatory mechanism, including specific CD4+ T cells and regulatory T cells.

V. CONCLUSION

We have found that IFN-γ was produced by CD8+ T lymphocytes against our HCV-specific epitopes in patients with SVR and NR. The IFN-γ productivity showed higher frequency in NR group than in SVR group to each HCV-specific epitopes (C36, C132,NS4 1789, NS5B 2510, NS5B 2588, C41, C110).

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Abstract (In Korean)

만성 C형 간염 환자를 대상으로 HCV-specific epitopes을 이용한 인터페론-감마 유도능과 그것과의 임상학적인 연관성

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이 명 은

만성 C형 간염 환자의 경우 약 50% 의 경우만이 인터페론/리바비린에 기초한 치료방법에 의해 효과를 나타내기 때문에 백신을 포함해 치료변화에 대한 요구가 중요시된다. 몇몇의 cytokines (IFN-γ) 은 면역반응의 조절과 만성 C형 간염 바이러스를 포함하여 바이러스와 관련되어진 질병을 조절하는데 있어서의 중요한 역할을 한다. 그러므로 만성 C형 간염 환자의 서로 다른 IFN-γ productivity 는 인터페론 치료를 극복하는데 있어 연관되어 있을 것으로 보인다.

따라서, 우리는 인터페론 치료에 의한 sustained virological responders (SVR) 과 non responders (NR) 환자들을 대상으로 HCV-specific epitopes을 이

용하여 그들의 IFN-γ productivity 대해 조사하였다.

만성 C형 바이러스 간염환자 40명을 대상으로 21명의 SVR 과 19명의 NR 환자로 구분하였다. 40명의 만성 C형 바이러스 간염환자로부터 그들의 말초단핵핵세포 (PBMC)를 ficoll을 이용한 원심분리기의 층 분리로 분리한 뒤에 각각의 peptide 와 인터루킨 15 (IL-15)을 처리하여 5일 동안 세포배양 한다. IFN-γ productivity은 ELISPOT 분석과 flow cytometry 방법에 의해 측정되었다. HLA typing 과 virus genotyping에 대한 측정은 각각 Micro SSP HLA class I DNA typing 과 Radioimmunoprecipitation (RIPA) assay 방법을 수행하였다.

만성 C형 바이러스 간염환자의 대부분은 다양한 HLA supertypes 을 갖고 있는 것으로 조사되었으며(HLA-A2 (27.5%), HLA-A3 (42.5%), HLA-B7 (30%)), 그들의 평균나이는 49.8세로 남녀간의 성 비율은 2.4:1을 보였다 (M:F=2.4:1). 우리의 결과는 HCV-specific epitopes 에 반응하여 CD8+ T cell에서 유도된 인터페론-감마의 IFN-γ productivity 가 NR 환자의 경우 SVR 과 비교하였을 때 높게 나타났다.

결론적으로, 이러한 HCV-specific epitopes 이 만성 C형 바이러스에 감염되고 인터페론치료에 대한 반응이 없던 NR 환자의 저하되어 있는 세포성 면역반응을 유도하는 새로운 치료로써 이용될 가능성이 있다는 사실을 확인하였다.

핵심되는 말 : C형 간염 바이러스, HCV에 특이적인 epitopes, IFN-γ productivity sustained virological responders (SVR) , non responders (NR) 인터페론