



Journal of Medical Sciences

ISSN 1682-4474

science
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Research Article

Level of Interferon-gamma and Interleukin-12 in Several Genotypes of HCV Infections

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Abstract

Background and Objective: The genotypes of HCV display different geographic distribution in worldwide, also as a marker of immune response to antiviral therapy and serves as a guideline for the duration of therapy. The outcome of HCV infection is determined by the host immune response, eradication of HCV depending on rapid induction of immunity. The failure to produce an effective immune response during acute infection is a key factor in the development of chronic hepatitis. This study aimed to investigate the correlation between genotype specific region of HCV in West Sumatra toward the level of gamma (INF- γ) and interleukin-12 (IL-12). **Materials and Methods:** This study combined the molecular examination for determining the genotype of hepatitis C virus and ELISA method for investigating the level of INF- γ and IL-12 in blood serum of patient with HCV infection. The data were analyzed by using Student t test. **Results:** A total of 75 samples, only 53 samples were successfully sequenced in the region of NS5B. The result of study found three different genotypes of HCV (Genotype 1, 2 and 3). The highest frequency of HCV genotype was Genotype-1 (N = 39, 73.58%) and subtype-1a was the most HCV subtype was frequently found. The average of INF- γ level in HCV genotype 1, 2 and 3 was 10.85, 8.75 and 10.56 pg mL⁻¹, respectively. The average of IL-12 level was 4.08, 5.00 and 3.64 pg mL⁻¹, respectively. This study found that there were no significant differences of INF- γ and IL-12 levels in HCV genotype 1, 2 and 3. **Conclusion:** The level of INF- γ and IL-12 did not differ significantly in HCV genotype 1, 2 and 3. HCV genotype-1 was the most genotype found in all sample.

Key words: HCV, interferon-gamma, interleukin-12, genotype, subtype 1a

Citation: Almurdi, Ellyza Nasrul and Saptino Miro, 2018. Level of interferon-gamma and interleukin-12 in several genotypes of HCV infections. J. Med. Sci., 18: 180-185.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The HCV is a major health care problem worldwide, infection with this virus can either lead to spontaneously or develop into chronic liver disease. Although the infection can be eliminated, most of patients with HCV develop to chronic disease. The total recovery of HCV is rare in the chronic phase of infection. HCV infects about 3% of world's population and WHO estimates that approximately 200 million people worldwide are infected with HCV¹⁻⁴.

The HCV is a single stranded RNA virus, 50 nm in size, spherical, enveloped. It is a member of the genus *Hepacivirus* in the family *Flaviviridae*. The virus has 9600 nucleotides, consists of 3000 amino acids, divided into 6 major genotypes (genotype 1-6) and more than 80 subtypes. HCV genotypes 1, 2 and 3 have a broad geographical distribution, whereas HCV genotypes 4, 5 and 6 are generally restricted to specific regions. Genotype-4 is found in North Africa and in Middle East. Genotype-5 and 6 is found in South Africa and Hongkong^{5,6}.

Previous studies of HCV have been performed by El-Bandary *et al.*⁷ in Egypt to identify the polymorphism of IFN- γ gene associated with the sensitivity toward HCV infection. The novelty of this study was to compare the level of IFN- γ and IL-12 in various genotype of HCV genotypes and investigate the correlation between HCV genotypes toward IFN- γ and IL-12. It was important because different HCV genotypes also have different responses to antiviral drugs, different disease progressions and different viral load counts.

In Indonesia, previous study conducted in several hospitals in Jakarta found that HCV genotype-1 was frequently found in patients, followed by genotype-2 and genotype-3. HCV genotype-1 is found about 46% of all infection in the world, followed by genotype-3, 2 and 4 (22, 13 and 13%)⁸. Genotype analysis in West Sumatra found three different genotypes of HCV (genotype 1, 2 and 3) and genotype-1 was the most prevalent genotype found⁹. The target site in the conservative region to determine the HCV genotype is the region of 5' UTR, core region, envelope and NS5B. The target site can detect HCV genotypes and subtypes^{10,11}.

The identification of HCV genotypes is important for patient management, determination of therapy duration, response to antiviral and geographic distribution. Various examination methods to determine the viral genotype can be performed through restriction fragment length polymorphism (RFLP) method, using specific primers and hybridization with specific probes. The current method for identifying the viral genotypes can be determined by direct sequencing^{12,13}.

The outcome of HCV infection is determined by the host immune response. The eradication of HCV depends on rapid induction of immunity, especially the interferon inducing the genes, interferon is released by dendritic cells, serves as potent antiviral activity and also supports the subsequent steps of antiviral immunity such as the activation of natural killer (NK) cells and cytotoxic T cells (Tc)^{14,15}.

When the virus particles infect the liver, APCs (antigen presenting cells) such as macrophages in peripheral circulation and local Kupffer cells produce IL-12 to initiate specific differentiation of the T helper (Th) naïve cells into Th1 cells. The activated Th1 cells will secrete the IFN- γ and IL-2 to induce the activation and proliferation of CD8 T and NK cells which play a role in viral eradication^{16,17}. The aims of the study were to identify the viral genotypes by direct sequencing and to measure the level of IFN- γ and IL-12 in several genotypes of HCV in patients with HCV infection.

MATERIALS AND METHODS

The study was performed on June, 2014-December, 2015. Samples were collected in all branches of Red Cross Indonesia and in Clinical Pathology Laboratory, M. Djamil General Hospital. The analysis of ELISA was conducted in Regional Health Laboratory, Padang. Molecular analysis was conducted in Biomedical Laboratory, Faculty of Medicine, Andalas University, Padang, Indonesia.

Preparation of samples: Blood serum were collected from 75 participants consisting of blood donors and from patients who visited in clinical pathology laboratory for anti-HCV positive diagnosis using third-generation HCV enzyme immunoassay (Abbot Laboratories, USA). Plasma or serum was separated from whole blood and stored at -80°C. This study was approved by Ethics Committee of Medical Faculty, Andalas University, Padang, Indonesia.

Viral RNA extraction and RT-PCR: The HCV RNA was extracted from 140 μ L blood serum using QIAamp viral RNA mini kit (Qiagen, USA) according to the manufacturer's protocol and stored at -80°C until further analysis.

The RT-PCR was performed using one-step RT-PCR kit (Invitrogen, USA). The NS5B region was amplified by PCR with NS5B-1 primer (forward): 5'-TATGAYACCCGYTGCTTTGAC-3' and NS5B-2 primer (reverse): 5'-GAGGAGCAAGATGTTATCA GCTC-3'¹⁶. DNA amplification was performed with reaction conditions were as follows: 95°C for 3 min followed by 40 cycles at 94°C for 15 sec, at 55°C for 30 sec and at 72°C for 1 min in a thermal cycler (Biorad, USA). The last cycle was followed by a 5 min extension step at 72°C.

One microliter of amplicon was used for second-round PCR using Go TaqGreen (Promega, USA). The following cycling parameters were used for 35 cycles. The amplified products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The gel was visualized under an ultraviolet trans-illuminator with a 100-base pair (bp) ladder (Promega, USA) and photographed.

DNA purification and sequencing: Amplicon was purified by using the QIAquick PCR purification kit (Qiagen, USA) and visualized by ethidium bromide 2% agarose gel electrophoresis for last verification. Samples were sent to Macrogen, Korean Republic for DNA sequencing analysis.

Alignment of sequence target: The sequence of DNA was edited using Geneious's version R 7.

Identification of IFN- γ and Il-12 levels: The IFN- γ and Il-12 levels were identified based on ELISA sandwich. Briefly, 100 μ L blood serum were transferred into wells of microtiter with anti-HCVc antibody which were already coated in plate, incubated for 2 h and wash five times with 250 μ L wash buffer. About 100 μ L FITC-Conjugated anti HCHcAg were added to each well, incubated and wash, 100 μ L

HRP-conjugated anti-FITC monoclonal antibody was added into wells. About 100 μ L substrate solution was added and stopped the enzyme reaction by adding the stop solution. The absorbance of each well was measured using ELISA reader at 450 nm (R and D systems).

Statistical analysis: Data were analyzed using SPSS ver. 15.0 software, the frequency of samples was presented as means. Data were compared using Student t-test. The p-values < 0.05 were considered statistically significant.

RESULTS

In this study, a total of 75 samples, 53 samples (70.67%) showed positive HCV after amplification. The HCV genotyping using NS5B sequencing obtained good quality of DNA. Result of electrophoresis showed the product of DNA fragment was 449 bp in position (Fig. 1).

A total of 53 samples with positive HCV, only 50 samples could be grouped individually into genotype-1, 2 and 3 it is shown in Table 1. Most of samples was predominantly included into genotype-1 (73.58%), followed by genotype-3 and genotype-2 (16.98 and 3.77%, respectively). Genotype-1 consist of three subtypes (1a, 1b, 1c), genotype-2 only has

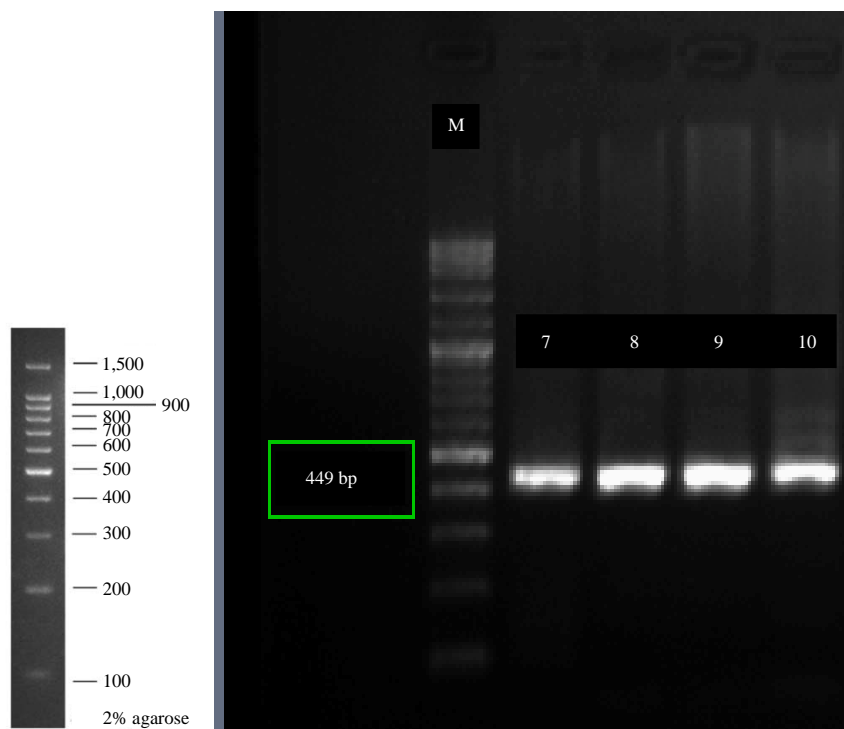


Fig. 1: Electrophoresis result of NS5B PCR products on 2% agarose gel. The amplified DNA band size was 449 bp (M: 100 bp DNA ladder, 7-10: Samples)

Table 1: Frequency of HCV genotype in NS5B target site

Genotype	Subtype	No. of samples	Percentage
Genotype-1	1a	27	50.94
	1b	5	9.43
	1c	7	13.21
		39	73.58
Genotype-2	2e	2	3.77
Genotype-3	3a	5	9.43
	3k	4	7.55
		9	16.98
Unidentified		3	5.66
Total	-	53	100.00

Table 2: Level of IFN- γ and IL-12 (pg mL⁻¹) in several genotype of HCV infection

HCV genotype	n	IFN- γ	p-value	IL-12	p-value
1	39	10.85	0.917	4.08	0.688
2	2	8.75		5.00	
3	9	10.56		3.64	

a subtype (2e) and genotype-3 has two subtypes (3a and 3k). The highest frequency of subtype was 1a subtype (n = 27, 50.94%) and the lowest frequency was 2e subtype (n = 2, 3.77). This study also found unidentified samples (n = 35.66%) and genotyping was discontinued.

The data in Table 2 showed that the most prevalent genotype in this study was HVC genotype-1 (n = 39), followed by genotype-3 (n = 9) and genotype-2 (n = 2). The higher level of IFN- γ was in genotype-1 (10.85 pg mL⁻¹), whereas. the higher level of IL-12 was in genotype-2 (5.00 pg mL⁻¹). The level of IFN- γ and IL-12 did not differ significantly between HCV genotype 1, 2 and 3 (p = 0.917 and p = 0.688). Each genotype result in not much different level both IFN- γ and IL-12.

DISCUSSION

The identification of HCV genotype is not only associated with the geographic distribution, but also related to the therapeutic response and the determination of therapy duration. Patients with HCV-1 infection have a lower rate of sustained immune response than patients with HCV-2. HCV-1b has a higher risk for developing into hepatocellular carcinoma compared with other genotypes. The role of genotypes in the progression of HCV infection and liver disease is well documented, for examples HCV genotype-1b and 3 have been reported to relate with more severe liver diseases¹⁸. The response of treatment to patients with HCV genotype-2 and genotype-3 was approximately 88.0%, whereas patients with HCV genotype-1, genotype-4, genotype-5 and genotype-6 have 48.0% of response to therapy^{19,20}.

In this study, HCV subtype-1a was the largest subtype among other subtypes (n = 27, 50.94%). The results of study were in line with Anggorowati *et al.*²¹ found that the HCV

subtype-1a was more often found (52.0%) than other subtypes. Prasetyo *et al.*¹⁰ found that HCV subtype-1a was 46.7%, subtype-1c was 16.7% and subtype-1b was 3.3%, revealed that the percentage of subtype-1b decreased as the previously dominant subtype in Indonesia. It also occurs in some cities such as Manila, Hanoi and Ho Chi Minh city which formerly dominated with HCV subtype-1b. In this study, HCV subtype-1a frequently found than subtype-1b²².

The HCV subtype-1a and subtype-3a are often associated with intravenous drug users, whereas, subtype-1b is related with blood transfusion or nosocomial infection. The infection with HCV genotype-2 is connected with geriatric patients²³. The use of needles together has become the primary transmission route in industrial countries, while blood transfusion remains as a source of contamination in developing countries, as the blood screening is not widely implemented²⁴.

When the HCV particles invade the liver, the APC produces IL-12 which specifically differentiates into the naïve CD4 Th cell into Th1 cells. Activated Th1 cells secrete IL-2 and IFN- γ induces the activation and proliferation of CD8 T cells and NK cells to eliminate the viral infected cells²⁵. Interferons are the central cytokines which responsible to induce an antiviral in cells and to activate and regulate the cellular components of innate immunity such as NK cells²⁶. The IFN- γ is produced by NK and NK T-cells as a part of the innate immune response and by antigen-specific T cells (both CD4 Th1 and CD8 T cytotoxic T lymphocytes). These cytokines are essential for the defense against the virus and acts on multiple cell types including immune cells²⁷. The CD8 T-cells which capable of secreting perforin and granzyme, are responsible for destructing of viral infected the cells.

Interleukin-12 has been recognized as an important cytokine linking of innate and adaptive immunity²⁸. The biological function of IL-12 are pivotal in both innate and adaptive immunity, it due to induce IFN- γ production from CD4 T-cells, NK cells, NK T-cells in early phase of the immune response and induce the differentiation of CD4 T-cells in Th1 effectors²⁹.

This study found no significant differences between IFN- γ and IL-12 levels toward HCV genotype infection. Besides, IFN- γ and IL-12 levels varied between three genotypes (Genotype 1, 2 and 3). Several factors influence the eradication of virus (such as virological characteristic, host immunity, genetic factors) and also determine the disease progression. The HCV also capable to subvert the immune functions including the NK cells, dendritic cells and T cells. This virus can alter their antigenic epitopes and escape from immune surveillance.

The failure of body to produce an effective immune response during acute infection is a key factor in the development of chronic hepatitis. In patients with chronic hepatitis C, acquired CD4 T cell and CD8 T cell decline. Inefficient Th1 cell response was marked by the loss of secretory capacity of IFN- γ caused by wrong differentiation of CD4.

CONCLUSION

The IFN- γ and IL-12 levels varied between three genotypes (Genotype 1, 2 and 3), but the level of IFN- γ and IL-12 were not statistically significant to every genotype of HCV. The results indicate that the HCV genotypes are not the predictor to determine the severe of HCV.

SIGNIFICANCE STATEMENT

Study on genotypes of HCV toward the level of IFN- γ and IL-12 was performed to continue the previous study about the distribution of HCV genotypes in West Sumatra. The HCV are divided into six genotypes distributed largely in worldwide. Most of genotype is spreaded in specific region. The identification of HCV genotypes is important for patient management, determination of therapy duration, response to antiviral and geographic distribution. NS5N is a conservative region to detect HCV genotype. This study found that HCV genotype-1 was the most prevalent genotype than genotype 2 and 3, with 1a as prevalent subgenotype. But, there were no significant differences in IFN- γ and IL-12 levels between three genotypes.

ACKNOWLEDGMENT

The authors thank to The Ministry of Research and Technology through DIPA fund of Andalas University for financing of fundamental research in 2016 with contract number: 97/BPPT/PNP-FK-Unand-2016.

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