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Study the genetic variation of KIR genes in patients with HCV in Wasit Province/ Iraq

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Abstract

One of the global public health diseases is the hepatitis C. It is widely distributed and primarily begin with acute infection. Some of them spontaneously clear the virus by their immune system. However, more than half of HCV infected individuals will develop their infection to chronic. No effective vaccine has been discovered against HCV until know. In most patients with 8-12 weeks of all oral drug regimens, direct-acting antiviral treatment will result in a virologic cure. Since the virus targets the immune system, study of KIR genes is involved in this research. Detection on these genes conducted by qPCR technique. This work aimed to detect KIR genotypes in patients with HCV compared with healthy subjects and to find out the effect of each gene as an indicator of the decline or progression of the infection. The results showed BX genotype was more frequent in patients, KIR2DL4 and KIR2DL5 genes were frequent in patients more than in control. However, KIR2DL1, KIR2DS2, KIR2DS3, KIR3DL2, KIR2DS1, KIR2DS4 and KIR2DL5 were significantly frequent in patients with HCV (RNA+). In conclusions; genes of KIR2DL1, KIR2DS2, KIR2DS3, KIR3DL2, KIR2DS1, KIR2DS4 and KIR2DL5 considered as risk factors for persistent and leading to the late infection.

Keywords: HCV, KIRs.

Introduction

Hepatitis C-virus is one of the public health disorders in Iraq and other countries in the world. It causes acute hepatitis and may extend to chronic infection, if it is untreated or progress which may leading to hepatocellular carcinoma and even death. The virus of hepatitis C is a blood-borne and globally infect 71 million individual with chronic cases (Moosavy *et al.*, 2017). The highest prevalence of HCV infection was between (6-28%) in Egypt. However, HCV is considered low-endemic in Iraq, with a prevalence of (0.5%) in a blood donor peoples (WHO, 2008). Fever, nausea, fatigue, abdominal pain and other symptoms appear in people who are acutely infected. Detection of anti HCV-antibodies performed in serological test, however qPCR technique is used to confirm the chronic infection by detecting on viral nucleic acid (RNA). Pathogenesis of this disease represented by viral positive stranded RNA genome, which is typed as *Flavivirus*. Primarily, this virus infects liver cells, and then establishes a persistent infection in the liver, leading to the progression of chronic hepatitis, cirrhosis and malignant of the liver cells, even though a few numbers of peoples are able to control the replication of virus spontaneously (Zaltron *et al*., 2012). The transmission of Hepatitis C is occurred firstly via the skin or from unsafely use of injected needles that contaminated with HCV, or by sexual and vertical contact that causing little spreading of the virus, this virus access directly to the liver cells, that are considered as major target (Belouzard *et al*., 2017).

Therefore, HCV is usually persist in the body unless attacked by Direct Acting Antivirals (DAAs) treatment (Smolders *et al*., 2019). No vaccine has been discovered until now against the virus. Liver damage or developed infection could be driven according to the immune response of the host (Guidotti and Chisari, 2006). Many of researches focused on the T-cells ability in fighting the virus. Most common type of these cells are (NK-cells) that considered essential cells for effective clearance of virus through an acute HCV (Shoukry *et al*., 2003). The activity of NK cell is determined by the equilibrium of receiving signals various and the balance between the activation and inhibition of the receptors; these receptors are known as "Killer cell Immunoglobulin-like Receptors (KIRs)" (Biassoni, 2008). Fourteen separated KIRs have been distinguished in this study; 8 inhibitory and 6 activating KIR types. The types and numbers of KIR genes substantially differ from one individual to another (Rajalingam, 2011) and the high diversity of *KIR* gene family is determined by variable gene content in haplotypes and allelic polymorphism (Anfossi *et al*., 2006).As many viruses, the development of HCV disease is associated with the genetic variation (Guo et al., 2019). Allelic polymorphisms are the main characteristics of KIR genes, this variation can influences on the NK cell's immune response through changing the selectivity of HLA and the affinity of ligands (Erken et al., 2015).

Materials and methods

This work was carried out in the University of Wasit, College of Science, Department of Biology and in the unit of Molecular & immunology at Al-Karama Teaching Hospital in Wasit Province/Iraq. Blood and serum samples were taken from 60 HCV patients (36 males and 24 females) with an age ranging between 16 and 75 years, as well as 29 samples used as control cases. Some clinical information or data were taken, involving the medical full history; complete of clinical tests and some other data such as age and gender of patients. However, other unavailable options were excluded, such as the stages of HCV infection and following up the line of treatment. Detection of HCV viral load in patients with HCV conducted by using qPCR technique after RNA isolation by using **(Quantiphor Viral RNA Extraction Kit. Cat. No: MB276v2f. Anatolia. Turkey. Bosphore®). Viral RNA Extraction Spin Kit is highly compatible with Bosphore® Real-Time PCR kits.** Starting sample volume is 400 µl and RNA recovery (elution) volume is 60 µl. qPCR viral load test performed by using (Bosphore® HCV Quantification Kit Cat. No: MB226v3f. Anatolia. Turkey). The protocol of the HCV quantification kit was composed of two steps of activation the HotStar Tag DNA polymerase by initial denaturation amplification cycle and a final hold. And two steps of amplification cycle and final hold. Table (1.1).

Table(1.1): HCV quantification PCR program.

Steps	Temperature	Time	No. of Cycles
Reverse Transcription	50℃	30:00 min.	1cycle
Initial Denaturation	95℃	14:30 min.	1 cycle
Denaturation	97℃	00:30 min.	40 cycles
Annealing(data collection)	55℃	01:20 min.	
Synthesis	72℃	00:15 min	
Hold	22℃	05:00 min.	1 cycle

The second step was extraction of DNA by using (Presto™Mini gDNA Extraction Kit. Geneaid Biotech Corporation. Taiwan). This kit was designed for 100 preparations, and was stored under standard conditions during working period.After that, qPCR technique was performed in presence of 14 genes of human KIR (28 forward and reverse primers) were typed as; inhibitory alleles including "KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR3DL1, KIR3DL2 and KIR3DL3" and activating alleles including; "KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1". All primers were supplied from Bioneer Company. The primers sequence showed in table (1.2) below, (Kulkarni et al., 2010).

Table (1.2): Sequences of primer that used for KIR genotyping

No.	Gene name	Sequence
1-	2DL1 F	GTTGGTCAGATGTCATGTTTGAA
	R	GGTCCCTGCCAGGTCTTGCG
2-	2DS2 F	TTCTGCACAGAGAGGGGAAGTA
	R	GGGTCACTGGGAGCTGACAA
3-	2DS3 F	TGGCCCACCCAGGTCG
	R	TGAAAACTGATAGGGGGAGTGAGG
4-	3DL2 F	CAAACCCTTCCTGTCTGCCC
	R	GTGCCGACCACCCAGTGA
5-	2DL2 F	CTGGCCCACCCAGGTCG
	R	GGACCGATGGAGAAGTTGGCT
6-	2DS5 F	AGAGAGGGGACGTTTAACC
	R	CTGATAGGGGGAGTGAGT
7-	3DL3 F	AATGTTGGTCAGATGTCAG
	R	GCYGACAACTCATAGGGTA
8-	3DS1 F	CATCGGTTCCATGATGCG
	R	CCACGATGTCCAGGGGA
9-	2DL4 F	TCAGGACAAGCCCTTCTGC
	R	GGACAGGGACCCCATCTTTC
10-	2DS1 F	TCTCCATCAGTCGCATGAG
	R	GGTCACTGGGAGCTGAC
11-	2DS4 F	GGTTCAGGCAGGAGAGAAT
	R	CTGGAATGTTCCGTKGATG
12-	2DL3 F	CTTCATCGCTGGTGCTG
	R	AGGCTCTTGGTCCATTACAA
13-	2DL5 F	ATCTATCCAGGGAGGGGAG
	R	CATAGGGTGAGTCATGGAG
14-	3DL1 F	CCATYGGTCCCATGATGCT
	R	CCACGATGTCCAGGGGA

qPCR technique was conducted by using (GoTaq® qPCR Master Mix kit. Cat. No:A6002. Promega Corporation. USA).

The total volume required for the reaction should be 20µl that consist of 10 µl GoTaq Mster Mix, 3 µl of 3 primers, 4µl of DNA template and 3µl of ddH2O.To start the reaction of PCR, first step was selection of SYBR® as detection dye for entire plate. As well as standard, two steps, 40 cycles and dissociation program were selected too. Table (1.3). The results of PCR product depended on dissociation curve analysis that also known as melting temperature (TM), by measuring the temperature during the separation of DNA strands in the point at which the dissociation of double strands DNA (dsDNA) reach to 50%. KIRs genotyping conducted by depending on TM of specific product. The TM value of each single primer Table (1.4) has been set by the qPCR method.

Table (1.3): qPCR program designed for KIR genotyping.

Steps	Temperature	Time	No. of Cycles
Hot Start Activation (Initial Denaturation)	95℃	2 minutes	1 cycle
Denaturation	95℃	15 seconds	40 cycles
Annealing/Extension	60℃	60 seconds	
Dissociation	60-95℃		1 cycle
Hold	40℃	5 minutes	1 cycle

Table (1.4): KIR primers with TM value

No.	Gene name	TM value
1-	2DL1	80.3
2-	2DS2	81.1
3-	2DS3	85.9
4-	3DL2	85.2
5-	2DL2	84.9
6-	2DS5	83.3
7-	3DL3	83. 7
8-	3DS1	82.7
9-	2DL4	84.3
10-	2DS1	81.0
11-	2DS4	83.1
12-	2DL3	80.8
13-	2DL5	85. 6
14-	3DL1	80.1

Statistical analysis

The statistical analysis of the results was conducted by the GeneStat Discovery program, Version 2012, Edition 4. t-Test, Mean, SD and SE were expressed in analysis of the data. Chi

square test used for the comparison of two groups (patients and control) with (KIR genes). The probability of ($p< 0.05$) and ($p<0.001$) was adopted to be a significant difference value.

Results

Patients samples involved 36 males (60%) and 24 females (40%), their mean age was 34.9 with a range of 16 to 75 years, compared with 17 males (58%) and 12 females (41%) of control group, their mean age was 42.8 with a range of 18 to 66 years, as shown in (Table 1.5).

Table (1.5): Characteristics of patients with HCV and control group

Characteristics	No. of Samples	Gender (n) (%)		Ages Min-Max / Mean±SD.	
HCV $^{+}$ (Patients)	60	Males	(36) 60%	(16-75)	34.9±14.1
		Females	(24) 40%		
Control	29	Males	(17) 59%	(18-66)	42.8±13.8
		Females	(12) 41%		

Ages of patients infected with HCV depend on indirect ELISA assay were taken randomly during collection of samples; these ages were classified to 6 groups as in table (1.6) as follows:(16-25) [n=8 (13.3%)], (26-35) [n=14 (23.3%)], (36-45) [n=14 (23.3%)], (46-55) [n=10 (16.7%)], (56-65) [n=12 (20%)] and (66-75) [n=2 (3.4%)]. Two groups (26-35) and (36-45) years were constituted the largest proportion and highly percentage [n=14(23.3%)], while lowest percentage was in group with (66-75) years old.

Table (1.6): Seroprevalence of HCV according to the age groups

Age groups	Number	Percentage
16-25	8	13.3%
26-35	14	23.3%
36-45	14	23.3%
46-55	10	16.7%
56-65	12	20%
66-75	2	3.4%
Total	60	100

A molecular assay based on HCV-RNA detection by quantitative real time PCR to find the concentration of viral RNA in the blood of patients and to confirmed the serological HCV test results, as well as this test used to assessment the chronicity of this disease and if it developed to late stages. The results revealed that (39) sample of patients were RNA positive (65%) while (21) were RNA negative (35%) from total numbers of 60 samples, as shown in table (1.7) below.

Table (1.7): Viral load assessment in patients with HCV

Viral load results	No.	Percentage
RNA+	39	65%
RNA-	21	35%
Total tested Samples	60	100%

Minimum value of viral load in patients with HCV RNA+ was (54 IU /ml) and maximum was (36,840,000 IU /ml). The HCV RNA+ patients were classified into three groups depending on viral concentration in their blood as following: low viremia if HCV RNA (< 400,000 IU/ml), intermediate if HCV RNA (400,000-800,000 IU/ml) and high represented by HCV RNA(> 800,000 IU/ml). This category was adopted according to Velosa *et al*., (2011).

In this study each one of 14 KIR genes was tested in 60 samples of patients with HCV and 10 samples of healthy cases as control and the total samples tests were 980. Therefore, from 840 samples only 483 KIR genes were present in patients, 227 were AA genotype and 256 were BX genotype. However in control group, from 140 samples only 94 KIR genes were present, 55 were AA genotype and 39 were BX genotype. Table (1.8).It is well known in the context that there is no clear criteria common or special of all haplotypes A and B can be distinguished; therefore the distinction of the genotype was based on classification, and each gene was analyzed separately to find out its role in the disease outcome. The method adopted in analyzing the data was in line with study of Hiby *et al*., 2010.

Table (1.8): KIR genotypes frequency in patients with HCV and control

KIR genotype in patients	**No.**	**Percentage**	**KIR genotype in control**	**No.**	**Percentage**
AA	227	47%	AA	55	59%
BX	256	53%	BX	39	41%
Total	483	100%	Total	94	100%

Each 14 KIR genes were detected in this study by count their frequency either in HCV patients (n=60) or in control group (n=10). Most of KIR genes were showed no significant differences in detection among patients and control cases. Except three genes were showed significant difference as ($p<0.05$) when they founded in HCV patients less than their founded in control group as following:

KIR2DS5 gene was observed in only (17) out of (60) patients while it observed in (6) out of (10) healthy cases. As well as KIR2DS4 was just found in (22) out of (60) patients but it found in (8) out of (10) control. Third one was KIR3DL1 gene that it detected in only (5) out of (60) patients but it was detected in (7) out of (10) of healthy group. However, two genes were showed significant difference as ($p<0.05$) by their detection more common in HCV patients than in control group as following; KIR2DL4 gene was more frequent in most of HCV patients (57) out of (60) than in control group (7) out of (10). Another one was KIR2DL5 gene that detected in (48) out of (60) patients but it detected in only (5) out of (10) normal cases. Table (1.9).

Table (1.9): Frequency of KIR genes in HCV patients and control

No.	KIR genotype	Frequency in patients (n=60)	Frequency in control (n=10)	X^2	P
1-	2DL1 AA	0.483 (29)	0.8 (8)	3.449	0.063
2-	2DS2 BX	0.800 (48)	0.6 (6)	1.944	0.163
3-	2DS3 BX	0.917 (55)	0.8 (8)	1.296	0.255
4-	3DL2 AA	0.850 (51)	0.9 (9)	0.175	0.676
5-	2DL2 BX	0.633 (38)	0.6 (6)	0.041	0.840
6-	2DS5 BX	0.28 (17)	0.6 (6)	3.896	0.048*
7-	3DL3 AA	0.67(40)	0.8 (8)	0.707	0.400
8-	3DS1 BX	0.233 (14)	0.4 (4)	1.246	0.264
9-	2DL4 AA	0.95 (57)	0.7(7)	6.836	0.009*
10-	2DS1 BX	0.600 (36)	0.4 (4)	1.4	0.237
11-	2DS4 AA	0.367 (22)	0.8 (8)	6.572	0.010*
12-	2DL3 AA	0.383 (23)	0.8 (8)	0.175	0.676
13-	2DL5 BX	0.800 (48)	0.5 (5)	4.195	0.041*
14-	3DL1 AA	0.083 (5)	0.7 (7)	22.948	0.000**

Each 14 genes of KIRs in this work were detected in 60 patients with HCV disease (39 RNA+) and (21 RNA-). From 840 samples only 483 KIR genes were present in patients from them 385 were detected in (RNA+) group, AA genotype were 166 and BX were 192 in number. Figure (3.7). However, only 125 KIR genes were present in (RNA-) group, 61 were AA genotype and 64 were BX genotype. Table (1.10) involved both groups.

Table (1.10): KIR genotypes frequency in (RNA+) and (RNA-) groups of patients with HCV

KIR genotype in (RNA+) patients	No.	Percentage	KIR genotype in (RNA-) patients	No.	Percentage
AA	166	46%	AA	61	49%
BX	192	54%	BX	64	51%
Total	358	100%	Total	125	100%

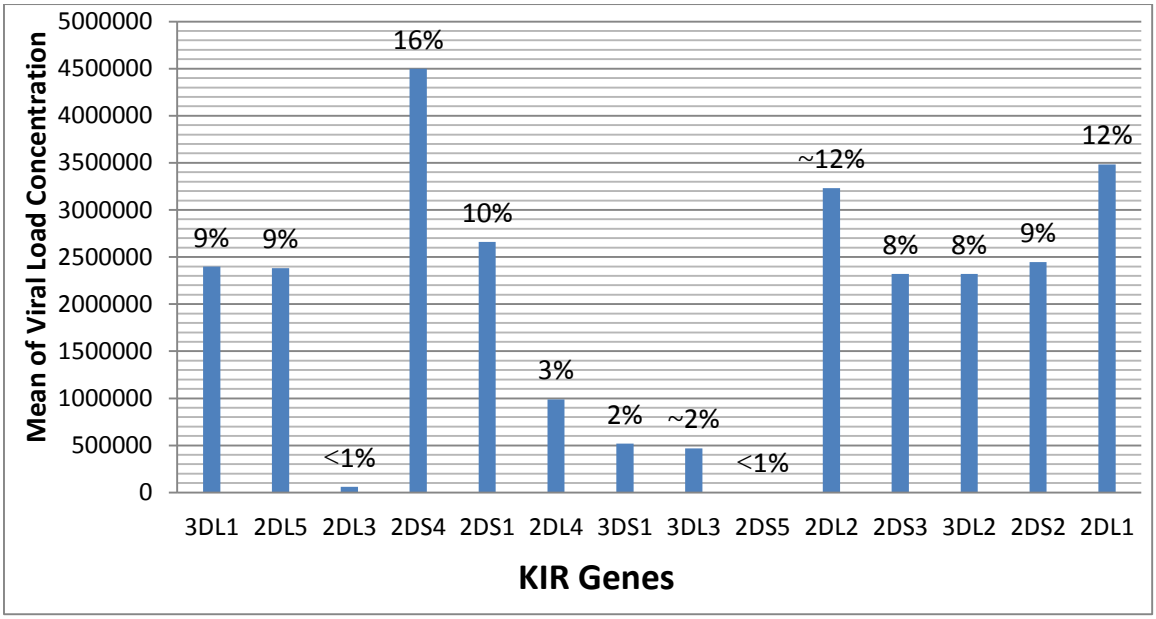
According to the single gene analysis, the results in the table below showed high significant difference (0.000) ($p<0.05$) for KIR2DS2, KIR3DL2, KIR2DS1 and KIR2DL5 when these genes detected in (RNA+) more than in (RNA-) HCV patients. The same thing with: KIR2DL1, KIR2DS3 and KIR2DS4, when these genes showed significant differences ($p<0.05$) at [(0.001), (0.001) and (0.038)] respectively. Otherwise, KIR2DS5 gene showed high significant difference (0.000) ($p<0.05$) when it detected in (RNA+) less than in patients with (RNA-) HCV. The remaining KIR genes didn't show any significant differences among two types of HCV patients. Table (1.11).

Table (1.11): Comparison among HCV (RNA+ and RNA-) patients according to KIR gene detection

No.	KIR Gene	Frequency in HCV PCR^+ patients (n=39)	Frequency in HCV PCR^- patients (n=21)	X2	P
1-	2DL1 AA	0.641 (25)	0.190 (4)	11.096	0.001**
2-	2DS2 BX	0.949 (37)	0.524 (11)	15.403	0.000**
3-	2DS3 BX	1.000 (39)	0.762 (16)	10.13	0.001**
4-	3DL2 AA	1.000 (39)	0.571 (12)	19.664	0.000**
5-	2DL2 BX	0.718 (28)	0.476 (10)	3.436	0.064-
6-	2DS5 BX	0.103 (4)	0.619 (13)	17.932	0.000**
7-	3DL3 AA	0.744 (29)	0.524 (11)	2.967	0.085-
8-	3DS1 BX	0.308 (12)	0.095 (2)	3.444	0.063-
9-	2DL4 AA	0.949 (37)	0.952 (20)	0.004	0.950-
10-	2DS1 BX	0.872 (34)	0.095 (2)	34.298	0.000**
11-	2DS4 AA	0.462 (18)	0.190 (4)	4.319	0.038*
12-	2DL3 AA	0.385 (15)	0.381 (8)	0.001	0.978-
13-	2DL5 BX	0.974 (38)	0.476 (10)	21.172	0.000**
14-	3DL1 AA	0.077 (3)	0.095 (2)	0.06	0.807

*Significant at ($p<0.05$), **Significant at ($p<0.01$)

Gene frequency of KIRs related with viral load was described by the calculation mean value of viral load concentration. KIR2DS4 was the more frequent gene in patients with highest viral load (4498911.39), (16%) followed by (12%) regarding KIR2DL1 (3484282.04) and KIR2DL2 (3229837.57) respectively. KIR2DS1was related with mean value as (2659686.79) at (10%). (9%) was the percentage of three genes; KIR2DS2 (2445530.76), KIR3DL1 (2399600.00) and KIR2DL5 (2381669.53). Both KIR2DS3 and KIR3DL2 genes have the same mean value in related with viral load (2320602.46), (8%). Others have low mean values with percentage (≤3%) comparing with previous genes. Figure (1.1).



Discussion

According to the gender of HCV infected individuals, this virus affects males more than females, and if they become chronically infected, women have slower rates of liver disease development than men , women are also more likely to spontaneously clear the virus (Baden *et al*., 2014). The current study was agreed with Fedeli *et al*., (2019) from Italy, who found the high distribution of HCV infection was in male 45% than in females 39%. In Iraq, Sharif *et al*., (2017) from Mosul, reported in their study the rate of HCV infection was higher in male than in female among hemodialysis patient (14%) and (6%) respectively. This also corresponds to the studies of Al-Zuheiry, (2016) from Diyala, and Mukharmash *et al*., (2017) from Wasit province. While current result disagreed with some studies also in Iraq; Jamil & Ahmad, (2015) from Baghdad, and Muslim, (2014) from Wasit province showed a higher percentage of HCV among females than in males. The vast majority of patients with HCV in this study were aged between 26 to 45 years with percentage of (23.3%), this outcome was agreed with the study of Petruzziello *et al*., (2019) when reported that the prevalence of HCV in the age range 31- 40 years was significantly higher in 2012 and above than in previous period. The study conducted by Saleh, (2012) reported that more than half of HCV patients were found to be in the third and fourth decades, i.e. 30-49 years, with 52.4% and 55.6% respectively for pre-treatment and post-treatment patients., this may also comparable to current data. Alao *et al*., (2009) showed the 41-50 age group had the highest seroprevalence of 10.9 percent , followed by 10-20, 21-30 and 31-40 age groups with a prevalence of 8.8 percent, 7.7 percent, 4.7 percent , respectively, while the above 50 age groups shows no indication of HCV antibodies. In study of Buseri *et al*., (2009); the highest rates of seroprevalence were found among the 18-47 years of age group. However, a lot of studies reported that HCV can infect people at any age but appears most common in adult especially the elderly above 50 years, but this statement not compatible with current study such as in study of Niu *et al*., (2016) which demonstrated that the highest distribution of HCV illness was at the age group 50-59 years. Hepatitis C virus RNA (viral load) is an important diagnosis of active HCV infection and clinically monitoring the body response to antiviral therapy (Kanwal *et al*., 2014). More precisely, viral load test Patients who spontaneously cured the infection and patients who developed chronic HCV were used to compare them. The viral load test outcome of current study compromised of 39 HCV RNA

positive and 21 RNA negative (out of 60 anti HCV patients) regardless acute or chronic cases, As it is difficult to assess the prevalence of HCV infection (i.e. the rate of newly acquired infections) since most acute infections are asymptomatic, available assays do not differentiate acute from chronic or resolved infection, and most countries do not routinely collect data on cases of acute disease, this clarification refers to another analysis; Alter, (2007). The observable RNA copies, therefore, indicate that these patients are either in acute or chronic stages. Undetectable findings, however, suggested that the virus was absent in the serum specimen of the patient. These individuals may spontaneously heal or undergo treatment after the acute HCV, then become SVR by clearing the virus, this possibility is clarified by Martin *et al*., (2014) when found in their study in chronic infected patients undergoing IFN-free therapy with a combination of faldaprevir (a NS3/4A protease inhibitor) and deleobuvir (a non-neocleoside NS5B inhibitor) with or without ribavirin, effective IFN-free therapy promptly restored the HCV-specific proliferation of CD8 + T cells (SVR results more than failure).
According to the patients group, the variation in presence of KIR genotypes was obvious when the rate of BX genotype was (61%) while AA genotype was (54%).During the continuous infection with HCV, the virus faces the pressure of immune cells over time by the activity of activating NK receptors that prevail against inhibitory receptors especially that have weaker inhibition such as KIR2DL3 toward activating receptors, this situation may causing accumulation of NK and CD+8 in parenchyma cells leading to damage the liver by enhancing the cytotoxicity and then exhaustion of T cells. This is what we found regarding the explanation why the presence of BX genotype was a little higher than AA genotype in patients.

In contrast with patients, AA genotype was a greater rate (78%) than BX (55%) in control group. To support current outcome, some of studies have a same views when it reported that activating receptors of KIR are lower affinities than inhibitory KIRs when they bind to their ligands, while their interaction to viral infection is highly relevant and the KIR B genotype in particular KIR2DS1 and KIR3DS1 association with chronicity and development of HCV, as well as and it is well established the drug of IFN-α which considered as standard therapy for HCV and HCV related lymphoproliferative occurrence and it may cause increasing NK cells cytotoxicity and activation (Valli-De Re *et al*., 2015; Malnati *et al*., 2017; Savoy and Boudreau, 2019).

At the level of genotyping among peoples, The diversity and biomedical importance of KIRs make it necessary for their organization to be studied. In addition to autoimmune diseases, there is growing evidence that receptor-ligand specificity among polymorphic KIRs and polymorphic MHC class I genes is associated with a wide range of infections, such as HIV and hepatitis C (Diaz-Pena *et al*., 2010). As well as, a lot of findings have shown that peoples vary in the type or number of KIR locus that contain (Jiang *et al*., 2012). The table above referred to fourteen KIR genes that detected in the HCV patients (RNA+ and RNA-) and compare the results with control group. According to the statistical analysis among patients and control on detection of KIR genes, KIR 2DS5 gene was appeared in few patients compared with healthy individuals with significant difference at (0.048) ($p<0.05$), this result showed that KIR2DS5 gene may expressed in healthy people or may also expressed in some patients who cleared the virus or respond to antiviral treatment, this explanation confirmed by the studies of Knapp *et al*., (2010) and Vidal-Castiñeira *et al*., (2010) when they founded the KIR2DS5 gene was related with viral clearance then it was also associated with sustain viral response (SVR). KIR2DS4 gene was also detected in few patients but more in control in current study with significant difference at (0.010) ($p<0.05$), this may also takes the same path as the previous result or there is another opinion as in the study of Middleton *et al*., (2007) when they observed that the KIR2DS4 gene is theoretically activating, but is disabled in approximately 75% of A haplotypes by a 22-bp frame shift deletion and is only functional in a minority of people. The

result of KIR3DL1 gene showed less frequent than other genes in HCV patients (5 only), but it was detected in most of control group. This gene was also detected by Podhorzer *et al*., (2018) in their study when they found Decreased expression of the KIR3DL1 gene in the presence of its ligand observed in peripheral NK blood cells from patients in the community of early CHCV infected patients who did not progress to cirrhosis suggests that decreased expression of KIR3DL1 may delay progression to cirrhosis. In study of Nozawa *et al*., (2013) In the Japanese population, a major association of this gene with SVR was also shown to be Peg-IFN / RBV.

Opposite of the previous results, the significant differences ($p<0.05$) at (0.009) and (0.041) showed by two genes (KIR2DL4) and (KIR2DL5) respectively, when these genes frequent in HCV patients more than in control group. With the regard of KIR2DL4, it is considered as reference gene because it expressed in all individuals either in patients or normal volunteers. The KIR2DL4 gene has unique properties: it codes for structural features that are characteristic of both inhibitory and activating KIR; it is expressed in all NK cells, whereas other KIRs are very clonally distributed on certain NK cells; and, unlike other KIRs that function as cell surface receptors, a soluble HLA-G molecular receptor is located in resting NK cells in endosomes where it can bind its ligand (Rajagopalan and Long, 2012). Few individuals had not expressed this genes in current study, this absence may belongs to samples type or laboratory circumstances may not completely appropriate. KIR, which has a stimulatory pattern and is involved in the clearance of HCV, may also play a key role in the treatment response. Whereas, KIR2DL5 was related to non-response (Carneiro *et al.*, 2010), this study is completely agreed with the current result of KIR2DL5 gene. The remaining KIR genes were found in different proportions in patients and healthy cohort; no significant ratios were found.
The results of this study indicated that the prolonged infection of the virus in patients with chronic phase of HCV, caused activation of NK cells regarding the activating NK receptors represented BX genotype. The validity of this interpretation is proven by recent finding of Oliviero *et al*., (2009) which reported that CHCV enhances the dominance of NK activating receptors. At the same context, CHCV patients with elevated levels of transaminase, their expression of activating receptors increased, such as KIR2DS3. Recent study of Podhorzer, (2018) established that the presence of KIR2DS3 in patients was also correlated with high or intermediate viral load. As well as, the study of Kusnierczyk *et al*., (2015) showed that KIR2DS3 increase in the absence of KIR2DS5 expression has been associated with low viral load. Thus what founded in current study because the KIR2DS5 gene was detected in (RNA+) group with low rate only in (10%) of patients, while KIR2DS3 detected in all (RNA+) patients and it recorded only (8%) in its association with their viral load. This elevated manner of this gene may predict the worsening of patient's condition to reach to the liver cancer and even lymphoproliferative disorders, as documented in the data of recent finding of Tanaka, (2019) which suggested that the KIR2DS5 gene may have a protective role against the progression of lymphoproliferative disorder in patients who have increase expression of KIR2DS3.
Detection of single KIRs gene frequency in HCV patients (RNA^+) comparing with (RNA^-) group was conducted by qPCR technique and the result of each gene will discuss as the following bellow:

1. **KIR2DL1**: The result showed the KIR2DL1 gene was found in only (25) out of (39) patients with positive RNA. However, this gene was found in (4) out of (21) RNA- individuals. There is obvious significant difference in number of this gene detection among HCV patients with positive viral load and in group of patients without viral load. This result gives possibility that KIR2DL1 gene has a strong inhibitory effect on NK cells to evade the virus in liver, as in other studies that showed the resemble results; Gomaa *et al*., (2012) In the persistent infection community, the incidence of KIR2DL1 increased significantly compared to that of resolved

HCV infection. Moreover, Vidal-Castineira *et al*., (2010) mentioned that KIR2DL particularly The ligand HLA genotypes (KIR2DL1) have at least one stronger inhibitory signal and their response to therapy has been less successful. As Peg-IFN- induces the expression of HLA class I molecules and favors the interaction between the infected cells and NK cells, this stronger inhibition could be strengthened by treatment. Likewise, the research of Megan *et al*., (2011) reported that KIR2DL1 gene increased in chronic HCV patients rather than resolvers but without significant value.

2. **KIR2DS2**: thirty seven (37) out of (39) patients with positive HCV RNA showed frequency in KIR2DS2 gene, while patients with negative HCV RNA showed this gene in only (11) from (21) samples, high significant appeared between these two groups with regard to KIR2DS2 gene frequency. KIR2DS2 is an antigen-specific receptor that recognizes retained Flaviviridae virus family peptides (Naiyer *et al*., 2017). Consistence with current result; the study of Vidal-Castineira *et al*., (2014) observed an association of *KIR2DS2*-HLA-C1 with NSVR. Others, such as Paladino *et al.*, (2007) and Dring *et al.*, (2011) found that Viral non-clearance in various populations turned out to be correlated with KIR2DS2. Marangon *et al.*, (2011) also showed higher frequencies of KIR2DS2 gene in HCV patients, but these researchers did not determine association of this gene with the development of disease.
3. KIR2DS3*:* high significant difference in this gene frequency when it was detected in all 39 HCV (RNA+) patients but it observed in only 16 patients with (RNA-).This result referred to that patients who have no viral load and express low level of activating compared to those who acquired a chronic infection, the KIR2DS3 receptor was capable of resolving the virus from their bodies. A significant correlation between NK cell-associated KIR2DS3 and chronic infection with HCV was also found in previous studies. In addition, this receptor has also been involved in multiple viral infections as a risk factor (Zhi-ming *et al*., 2007; Wauquier *et al*., 2010).
4. **KIR3DL2**: like KIR2DS3 gene, KIR3DL2 showed big significant difference in all patients with viral load comparing with patients who have no viral load. Two from most of previous studies supposed that KIR3DL2 must found virtually in all human (Jiang *et al*., 2012 and Marangon *et al*., 2014). Some of studies as of Gonzalez-Galarza *et al*., (2011) and Niepiekło-Miniewska *et al*., (2014) found the KIR3DL2 deletion was very rare in a few people, which is also what happened with the current research. As for HCV (RNA-) patients, some of them did not express this gene, this may be attributed that there was an association between haplotypes of KIR and chronic infection with HCV in case of KIR3DL2 and even KIR2DS3 frequency, this concept supported by recent study of Podhorzer *et al*., (2018) this confirmed the increased expression of KIR3DL2 in NK cells across the entire CHCV cohort of patients.
5. **KIR2DL2:** the presence of KIR2DL2 gene was not showed any significant difference between HCV (RNA+) and HCV (RNA-), and it was detected in each group with different numbers. This current outcome is comparable to what was founded by a few researchers about KIR2DL2 gene, as with Vidal-Castiñeira *et al*., (2014) when observed that this gene association with response to treatment and it appeared in people who have recovered from disease. While many other researchers confirmed that this gene was associated with chronic case and non-sustain viral response (NSVR) (Moesta *et al*., 2008; Vidal-Castiñeira *et al*., 2014; Vejbaesya *et al*., 2011 and Vasconcelos *et al*., 2013) and with HCC in young HCV patients (Podhorzer *et al*., 2018). The result of KIR2DL2 in this study may explain the group of chronic HCV may include small numbers of patients have sever progression in disease therefore no significant difference was found.
6. **KIR2DS5***:* this gene was detected in few numbers of HCV (RNA+) patient's group and was high in (RNA-). Therefore, the statistical analysis of this gene showed a high significant difference between two mentioned groups. This is agreed with the studies of Knapp *et al*.,

(2010) and Vasconcelos *et al*., (2013) when they discovered KIR2DS5 gene was not just related with viral clearance but also associated with SVR.

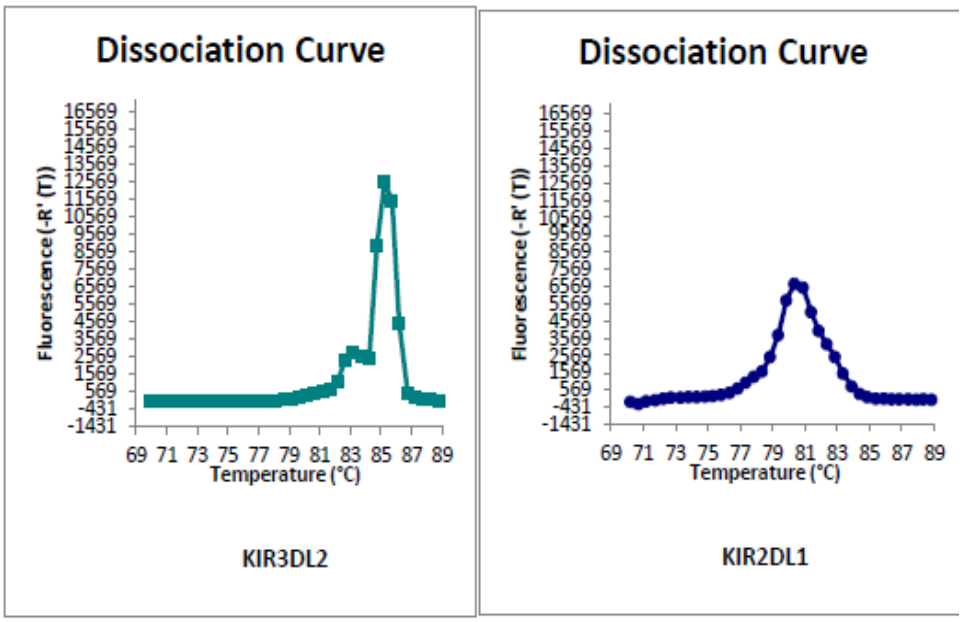
7. **KIR3DL3:** According to the most of studies, KIR3DL3 is supposed to appear in all people as in a framework genes (KIR3DL2 and KIR2DL4) but in current study this gene was absent in two healthy peoples and some of patients, no significant difference was shown between patients of (RNA+) and (RNA-) HCV, this is in line with previous researches of Torkar *et al*., (1998); Trompeter *et al*., (2005) and Leaton *et al*., (2019) that confirmed the low expression of KIR3DL3 on NK cells extracted from peripheral blood of adult human may attributed to methylation of the promoter. More information required about this gene because the function of this gene is not yet determined.
8. **KIR3DS1:** Statistical analysis of this gene did not show a significant value when comparing its presence in HCV (RNA+) and (RNA-) groups. Different views about KIR3DS1 frequency were found. Once, the expression of this gene was associated with the progression of HCV to HCC (Lopez-Vazquez *et al*., 2005) and another finding proposed the presence of KIR3DS1 give the patients with HCV protection from disease progression (Littera *et al*., 2013). In this study the KIR3DS1 frequency was distributed randomly in two groups of patients.
9. **KIR2DL4:** there is no significant difference when comparing KIR2DL4 in HCV (RNA+) and (RNA-) patients; because it is present at high levels in these two types of patients and it is assumed that it appears in all people as a reference gene. This gene was absent in only two people with HCV (RNA+) and one with HCV (RNA-), this result relatively resemble to this of Gonzalez-Galarza et al (2010) when it showed absent of KIR2DL4 in only three study cases, such genotype of KIR2DL4 and even KIR3DL2 gene in this finding and in current study are very rare in the world population (Ozturk *et al*., 2012).Therefore, more research on large number of population are required to determine the effect of deletions of these genes on health of individuals.
10. **KIR2DS1:** this gene was significantly raised in (RNA+) as compared to (RNA-) HCV patients in current study. KIR2DS1gene is express in world population with different frequencies depending on geographical distribution of the population with the range between 14-88%. Some genetic studies determine the comparison of activity of this gene between control cases and those infected with the virus, however the impact of it expression in infected people was not clearly defined (Wauquier *et al*., 2010 and Pydi *et al*., 2013).
11. **KIR2DS4**: A slight increase in this gene in patients with (RNA+) compared to (RNA-) with a significant level at (0.038) in this study. In general, this gene is often activating but it disabled by a frame-shift deletion (22-bp) in aproximately75% of individual (Middleton *et al*., 2007). In accordance with the current outcome, study of Podhorzer *et al*., (2018) found an increase in frequent expression of KIR2DS4 gene receptor on NK cells of CHCV patients who have high level of ALT. This increasing in expression may be a result of the low ability of acute HCV patients to resolve the virus as well as its presence in CHCV patients may cause damage of the liver parenchyma.
12. **KIR2DL3:** the result of KIR2DL3 gene revealed no significant differences among patients who have viral load and those who did not have. This result is acceptable according to other findings due to the wide variation in the genotype of this gene among individuals. One of the studies found KIR2DL3 was a good indicator of SVR and its related with treatment response, and the association with the therapeutic response may be greater when it interact with HLA-C1 ligand (McKiernan *et al*., 2004; Moesta *et al*., 2008 and Vidal-Castineira1 *et al*., 2014).Whereas the study of Arias *et al*., (2009) observed this gene was frequent in patients with cirrhosis. Another one found that KIR2DL3 has a protective effect during the initial stage of disease but not during overcoming of virus on patient's immunity (Ciccorossi *et al*., 2007).
13. **KIR2DL5:** Compared to the HCV (RNA-) cohort, the statistical analysis of this gene showed a high significant increase in patients with (RNA+). Increasing of this gene in patients with

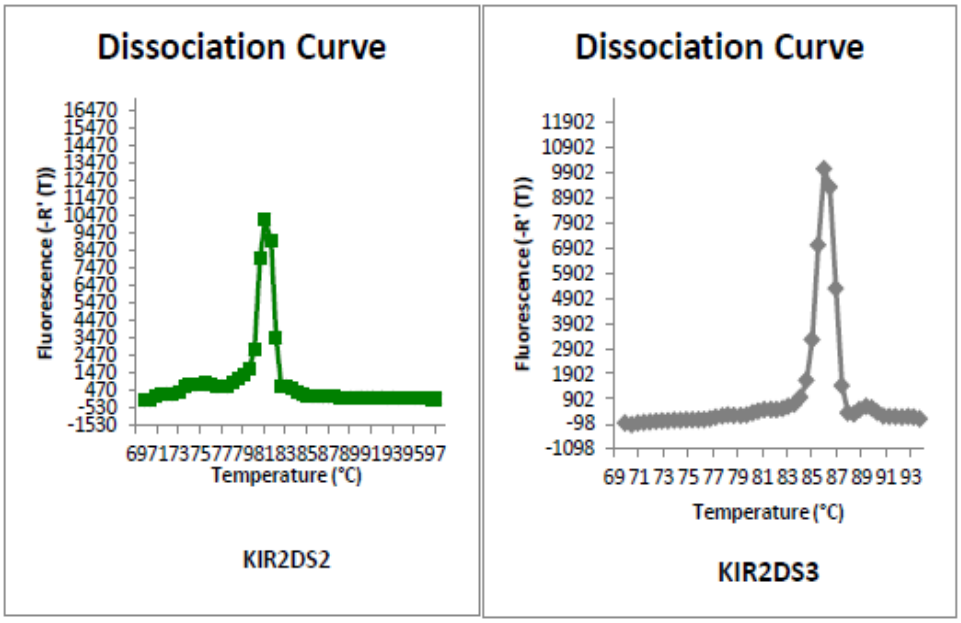
persistent infection may be consequence to poor response to treatment and without ability to spontaneous clearing the virus. In consistence with this idea, Carneiro *et al*., (2010) identified the association of inhibitory KIR2DL5 with NSVR. Resemble identification was documented by study of Vasconcelos *et al*., (2013).

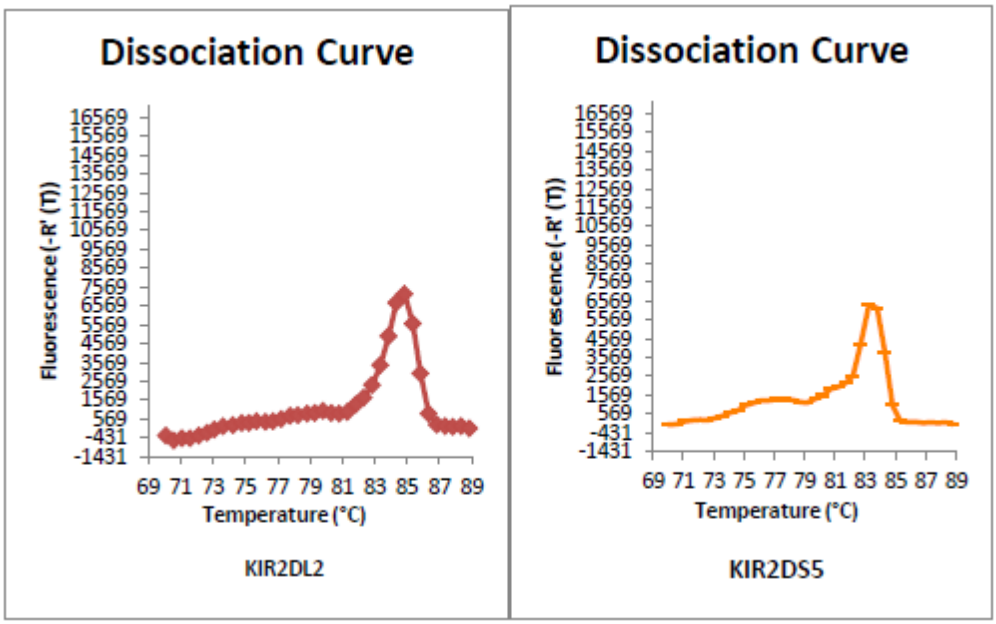
14. **KIR3DL1:** none significant difference was found the detection of KIR3DL1 in all patients either they did have viral load or did not have because this gene was missing in most of them. Even in other previous findings, it was detected in different groups of HCV patients, once KIR3DL1 detected in decrease expression in CHCV patients comparing with them in acute HCV stage, as study of Podhorzer *et al*.,(2018). Nozawa *et al*., (2013) showed the combination of KIR3DL1 with its ligand HLA-Bw4 was significantly associated with SVR in Japanese population. However the studies of Vidal-Castineira *et al*., (2010) and Knapp *et al*., (2010) did not find any association of this gene with SVR in Caucasian population.

The result of KIR2DS4 gene in this study recorded higher rate in its relation with viral load (16%). Depending on this result, it is likely that an increase of this gene frequency may be as a result of weak capacity of patient to get rid of virus in acute phase, thus the disease then progress and liver also damaged (Podhorzer *et al*., 2018). Moreover, and although not much existence of *KIR3DL*1 gene among people with HCV under study, it has been found to be associated with a high viral load. Due to small number of patients with HCV (RNA+) have KIR3DL1 in this study, this gene did not appear at a greater rate, so it can be said that the presence of these two genes may increase the problem of disease progression and may direct the CHCV towards cirrhosis or even liver cancer. This interpretation is fully consistent with result of (Stegmann *et al*., 2012 and Valli De Re *et al*., 2015) which found a higher frequency of KIR2DS4 and KIR3DL1linked with high viral load and in patients who have HCC. The second gene that has high percentage in its related with viral load comparing with the other genes was KIR2DL1. Few studies such as of Oliviero *et al*., (2009) and Holder *et al*., (2014) found the expression of KIIR2DL1gene was decrease in HCV infected patient and suggested it may regulate the chronicity and prevent the development of disease. The finding of Betser-Cohen *et al*., (2006) reported the presence of KIIR2DL1with MHC class 1ligand play an essential role in balancing the recognition of NK cells and then reducing inhibitory signals by decreasing the level of this gene during viral infection and tumor transformation. However, other studies (Gomaa *et al*., 2012 and Gardiner, 2015) confirmed that this gene abounds in chronic HCV patients, but without mentioning its relationship to viral load level. According to KIR2DL2 gene related with viral load in this study, the percentage was 12% as in previous gene, one possibility is that the activation of NK cells by releasing INF-α may be disrupted in presence of inhibitory KIR2DL2, which makes them lose the antiviral ability and then overcoming of disease. This view is agreed with Zwolinska *et al*., (2016) and Hu *et al*., (2018). When talking about KIR2DS1 gene related with HCV infection and viral load, the percentage was 10% in current study, this result showed that this activating gene associated with persistent infection and the role of it on controlling disease is not obvious, and not much finding of studies about this gene associated with viral disease were founded, this is also mentioned by study of Anais-Chapel, (2017) when it explained there is no viral peptide described to KIR2DS1 gene as well as, the functional importance of this genes unclear even in association with HLA-C2. Therefore, more studies are required about this gene. One activating KIR2DS2 and two inhibitory KIR3DL1 and KIR2DL5 genes were related with patient's viral load in 9%, while 8% for both KIR2DS3 and KIR3DL2 and others (≤3%). In agreement with current work; study of Paladino *et al*., (2007) also found reported that KIR2DS3 was correlated with high or intermediate viral load in patients. Whereas Pothorzer, (2018) reported that the expression of KIR3DL2 gene in CHCV patient may be indicated on disease progression to fibrosis. Study of Ursu *et al*., (2018) demonstrated that KIR2DS2 gene was associated with persistent of the virus in patients who could develop complications of this infection. Other one, Carneiro *et al*., (2010)

reported an association the presence of KIR2DL5 with viremia and responsiveness. Ultimately, it is necessary refer to recent information documented by Diaz-Peña *et al*., (2020) that despite all of previous speculations about the role of KIR genes variability in the development of HCV to hepatocellular carcinoma and even other cancers still unclear, this perhaps due to few studies including large groups of patients that well characterized.






Dissociation Curve
Fluorescence (-R' (T))
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569
-431
-1431
69 71 73 75 77 79 81 83 85 87 89
Temperature (°C)
KIR2DL2
Dissociation Curve
Fluorescence (-R' (T))
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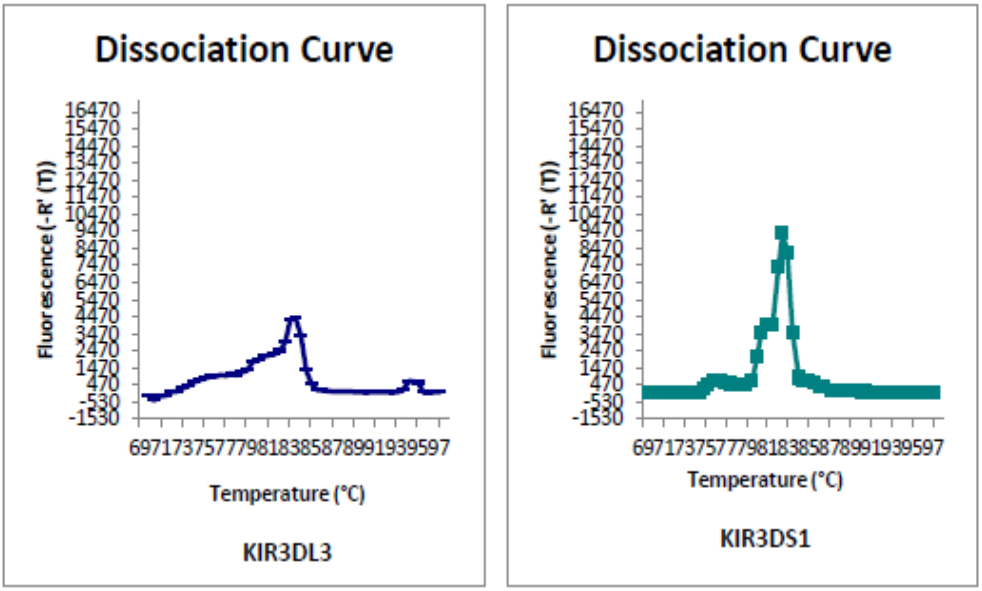
Temperature (°C)
KIR2DS5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Temperature (°C)
KIR3DL3
Dissociation Curve
Fluorescence (-R' (T))
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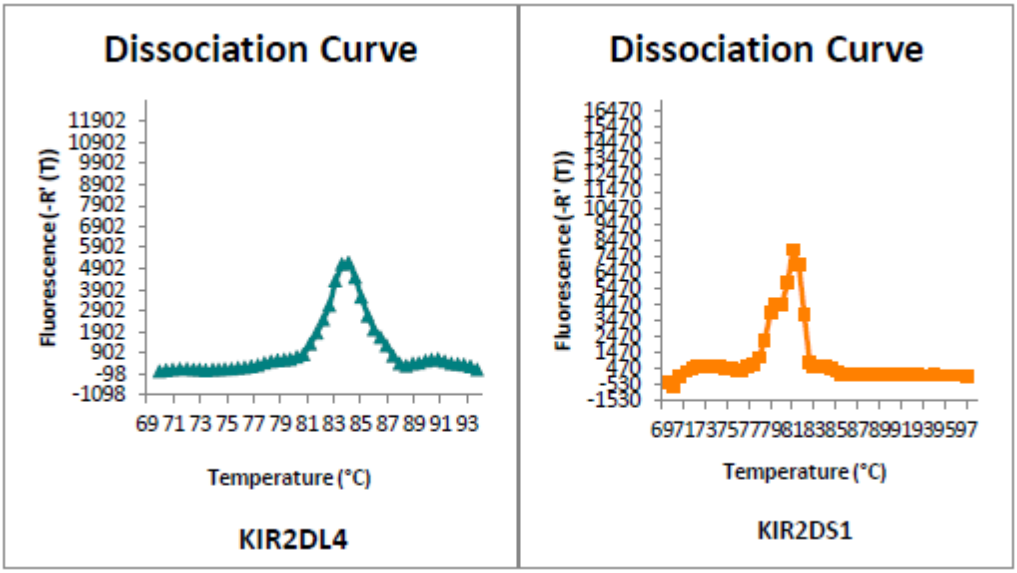
Temperature (°C)
KIR3DS1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Fluorescence (-R' (T))
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-1098
69 71 73 75 77 79 81 83 85 87 89 91 93
Temperature (°C)
KIR2DL4
Dissociation Curve
Fluorescence (-R' (T))
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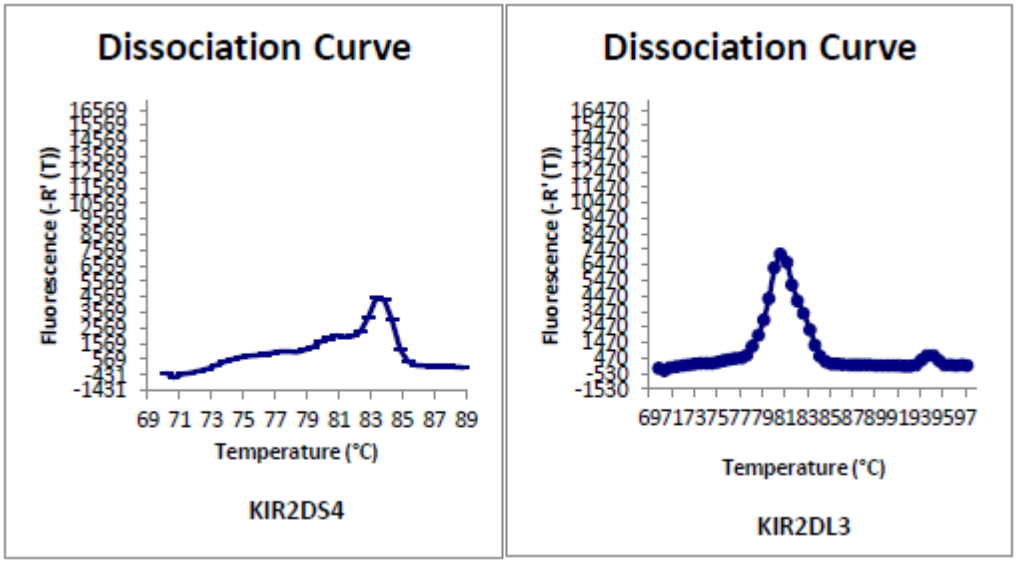
Temperature (°C)
KIR2DS1


Dissociation Curve
Fluorescence (-R' (T))
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Temperature (°C)
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Dissociation Curve
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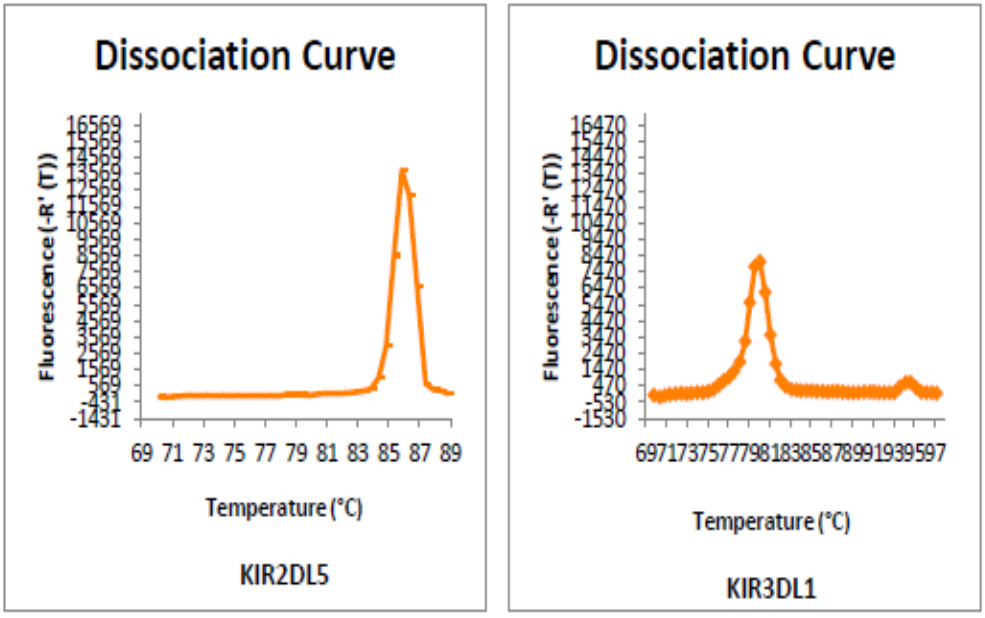
Temperature (°C)
KIR2DL3



Conclusions

Genes of KIR2DL1, KIR2DS2, KIR2DS3, KIR3DL2, KIR2DS1, KIR2DS4 and KIR2DL5 considered as risk factors for persistent and leading to the late infection of HCV. While, it can be consider that KIR2DS5 gene as an indicator for short period of infection. However, KIR2DS5, KIR2DS4 and KIR3DL1genes may have a protective effect against the virus.

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