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KIR Genotypes Impact Progression to Hepatocellular Carcinoma in Patients with Chronic Hepatitis C Infection

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Simple Summary: Egypt has the highest prevalence of hepatitis C infection in the world with a dramatic increase in the incidence of hepatocellular carcinoma (HCC) observed during the last decade. Currently, HCC is the most prevalent cancer in men and the second most prevalent cancer in women in Egypt. There are limited published data on the implication of host KIR genotypes and the progression of chronic hepatitis C virus (HCV) infection to HCC. We describe the first analysis of Egyptian KIR genotypes from the Nile Delta region. The findings of this study suggest that KIR haplotype AA, that contains dominantly inhibitory genes, was less frequent in HCC patients compared to chronic HCV patients and healthy control subjects. Therefore, haplotype AA may have a crucial role in host defense against HCC progression.

Abstract: In Egypt, hepatocellular carcinoma (HCC) is the most prevalent cancer in men and the second most prevalent cancer in women. In addition, Egypt has one of the highest prevalences of hepatitis C infection in the world. The aim of the present work was to study the potential role of the 16 KIR genes in the outcome of individuals with chronic hepatitis C virus (HCV) infection in Egypt. The study was carried out under an IRB-approved protocol. Sequence-Specific-Primer-PCR (SSP-PCR) was used for KIR genotyping of germline DNA extracted from peripheral blood leukocytes or from the non-tumor liver of 83 HCC patients, 100 patients with chronic HCV infection without HCC, and 120 matched healthy controls. Out of the 83 HCC patients, only 7 (8.4%) were treated by interferon and/or interferon Ribavirin combination, while for the remaining patients 50 (60.2%) received no prior HCV therapy and 26 (31.3%) were treated with direct-acting antiviral (DAA). Our results showed that KIR haplotype AA that contains more inhibitory KIR genes and fewer activating genes was observed with a significantly lower frequency in HCC patients (6/83, 7.2%) compared to chronic HCV (27/100, 27.0%) ($p = 0.0005$, OR = 0.21 [0.08–0.53]) and healthy controls (29/119, 24.4%) ($p = 0.001$, OR = 0.24 [0.09–0.61]). In addition, the frequency of genotype 6 (G6) which contains all the KIR genes was significantly high in the HCC patients (16/83, 19.3%) compared to chronic HCV (8/100, 8.0%) ($p = 0.02$, OR = 2.7 [1.11–6.79]) and healthy controls (8/119, 6.7%) ($p = 0.006$, OR = 3.31 [1.35–8.16]). Activating KIR genes 2DS1 and 3DS1 were significantly higher in HCC patients (48/83, 57.83% and 45/83, 54.22%) compared to the chronic HCV patients (36/100, 36% and 34/100, 34%), $p = 0.028$, 0.027, respectively. Our results are contrary to a prior work on HCC from patients with HCV who were mostly treated by interferon-based therapies. In conclusion, KIR haplotype AA has

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an important role in host defense against HCC progression especially in patients treated by DAA, suggesting an important role of the KIR genotype status on the outcome of chronic HCV infection.

Keywords: chronic hepatitis C; Egyptian; genotyping; hepatocellular carcinoma; KIR; SSP-PCR

1. Introduction

In Egypt, according to the most recent national survey of cancer, hepatocellular carcinoma (HCC) was the most prevalent cancer in males (33.6%) and the second most prevalent cancer in females (13.5%) with the Nile Delta region having the highest incidence of liver cancer (29.6%) [1]. In addition, Egypt has the highest prevalence of chronic HCV infection globally; with a seroprevelance of 7.5% in males and 5.3% in females [2], HCV infection represents the highest risk factor for HCC development in patients with cirrhosis [3].

The liver is highly enriched with innate immune cells, among which are natural killer (NK) cells that play a pivotal role in host defense against viral infection and tumor cells, and in initiating the adaptive immune response [4]. NK cells' function is controlled through an array of inhibitory and activating receptors expressed on their surface that interact with an array of ligands including human leucocyte antigen class1 (HLA class 1) molecules which are expressed on the surfaces of all nucleated cells. In humans, the most highly polymorphic of these NK cell receptors are killer cell immunoglobulin-like receptors (KIRs), with 14 KIR genes and 2 pseudo-genes [5]. These receptors regulate the function of NK cells and other lymphocyte subsets as natural-killer-like T-lymphocytes (NKT cells), thereby playing crucial roles in both innate and adaptive immunity [6]. KIR receptors are classified according to their extracellular number of immunoglobulin-like domains as 2D and 3D, then the cytoplasmic tail length as (L, S, and P for long, short, and for pseudo-genes, respectively), and sequence similarity characterized numerically by the last number to distinguish the structural similarity encoded by different genes [7]. The inhibitory KIR receptors have long cytoplasmic tails containing pairs of immune tyrosine-based inhibitory motifs (ITIMs), whereas the activating KIR receptors have short cytoplasmic tails that associate with the DAP12 signaling molecule via a positively charged lysine residue in their trans-membrane domain [8]. The aim of the present study was to investigate the potential role of KIR genes in the progression of chronic HCV infection to HCC in Egyptian patients. Our results suggest an important role of the KIR genotype status in the outcome of chronic HCV infection, especially those treated with direct acting antiviral agents (DAA).

2. Materials and Methods

2.1. Study Subjects

Study populations consisted of 303 Egyptians residing in the Nile Delta region. The study was carried out under an IRB (IRB no: 0051/2012)-approved protocol of the National Liver Institute (NLI), Menoufia University. All participants gave written informed consents for inclusion in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. The HCC and HCV patients were collected sequentially from the patients seen at the hepatology clinics, the National Liver Institute (NLI), Menoufia University. The control subjects were accrued from villages in the vicinity of NLI and from health staff at our institution who volunteered for the study through an independent protocol. The controls were age- and sex-matched to HCV and HCC subjects. For the HCC subjects, a total of 115 patients were accrued and tested in our laboratory for HBV and HCV markers. Exclusion criteria of HCC patients were: (1) Those with HBV infection; (2) Those with co-infection of HCV and HBV; (3) Those who were negative for viral markers. Out of the 115, 83 had HCV infection alone and were included in experimental work (n = 83). The participants included 83 patients with HCV-related HCC, 100 patients with chronic HCV without HCC, and 120 matched healthy controls with no HCV infection. The average age

of the three cohorts was 49.26 (range 21–76) with a median age of 50 years. The average age was similar (between the controls and those with HCV) but higher in the HCC cohort (Table 1).

Table 1. Demographic data of the studied groups.

Studied Variable	HCC $n = 83$	HCV $n = 100$	Controls $n = 120$	*p*-Value
Age				
Mean ± SD	56.21 ± 6.73	46.22 ± 6.53	46.97 ± 10.44	<0.001
Median	57.00	47.0	47.0	
Min–max	38.0–75.00	35.0–60.00	21.0–76.00	
Gender				
Female	13 (15.7)	21 (21.0)	45 (37.5)	0.001
Male	70 (84.3)	79 (79.0)	75 (62.5)	

At least 4 mL peripheral blood was collected in EDTA tubes from each subject. The blood was centrifuged at 2500 rpm to isolate the buffy coat containing peripheral blood leukocytes.

2.2. DNA Extraction

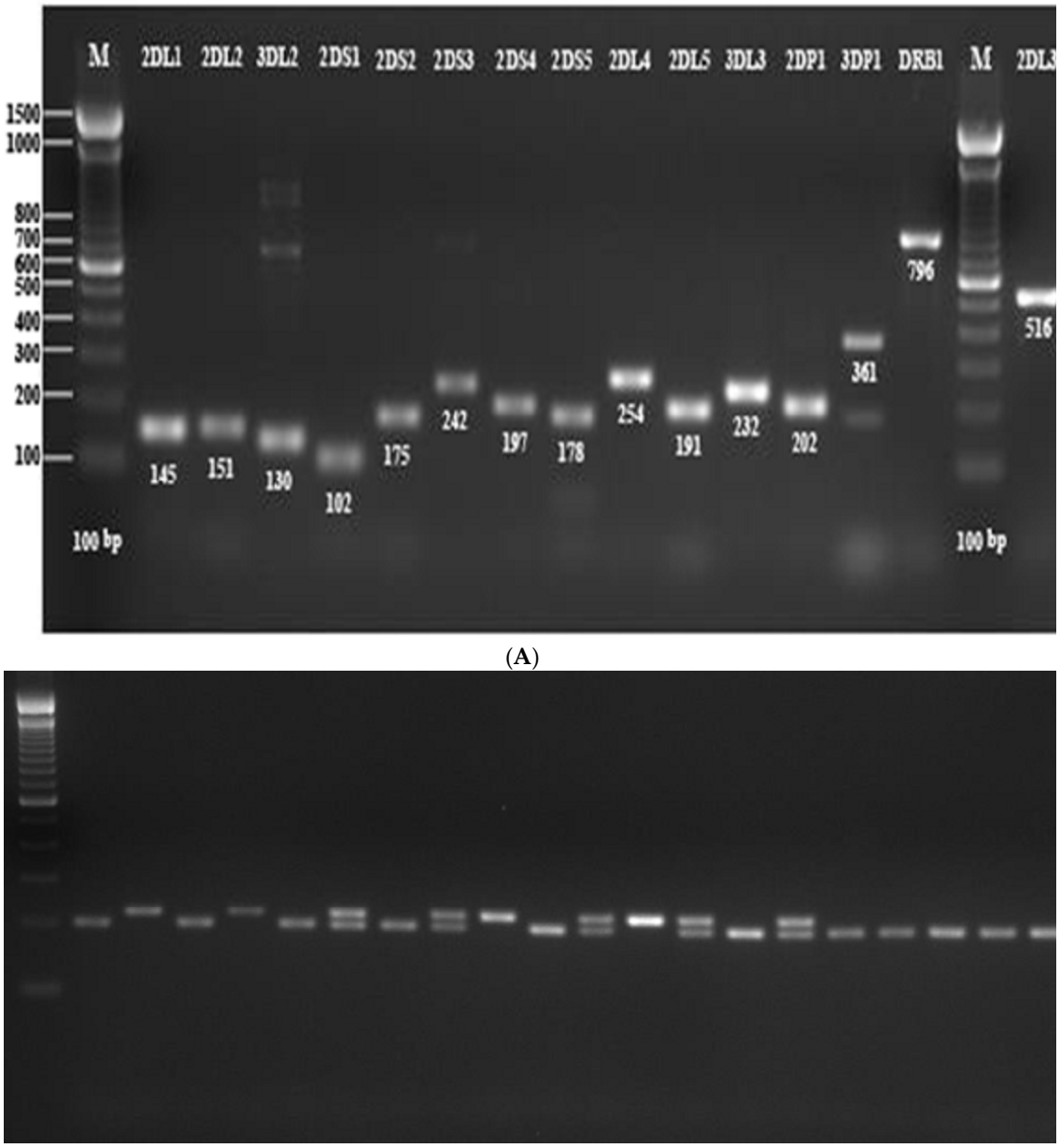
DNA extraction was performed from buffy coat for all control subjects, chronic hepatitis patients, and 36 out of the 83 HCC patients using QIAmp blood mini kit as per company instructions (Qiagen, Germantown, MD, USA). For 47 HCC patients with available tumor tissues, DNA was extracted from the fresh frozen non-tumor tissues using Gentra Puregene Tissue kit (Qiagen, Germantown, MD, USA). For all samples, the DNA concentration was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.3. KIR Genotyping

Sequence-Specific-Priming polymerase chain (SSP-PCR) genotyping was performed with modification from a published protocol, Figure 1 [9]. Briefly, one primer set with sensitivity higher than 96% http://www.ebi.ac.uk/ipd/kir/probe.html (accessed on 15 May 2023) was used for each KIR gene. Two sets of primers were used for KIR2DL3, KIR3DL1, and KIR3DS1. For all negative PCR results, the second primer of KIR gene was used to confirm the results. For KIR3DP1, the primers described by Gómez-Lozano N. and Vilches C. were used [10]. All primers were obtained from Integrated DNA Technology (Coralville, IA, USA). Primers were used at 10-pmol concentration per reaction. The SSP-PCR technique was performed using GoTaq green master mix (Promega, Madison, WI, USA) in a total volume of 10 µL. The PCR cycling condition was: denaturation at 94 °C for 3 min, 5 cycles (94 °C 15 s, 65 °C 15 s, 72 °C 30 s); 21 cycles (94 °C 15 s, 60 °C 15 s, 72 °C 30 s); 4 cycles (94 °C 15, 55 °C 1 min, 72 °C 2 min), with a final 7-min extension step at 72 °C using the GeneAmp® 9700 thermal cycler (Applied Biosystem, Drive Foster City, CA, USA). The PCR products were electrophoresed using 2% agarose gel at 100 Volts for 30 min, visualized using ethidium bromide stain and documented using UVITEC gel documentation system (UVITEC, Cambridge, UK).

2.4. KIR2DS4 Analysis

KIR2DS4 gene variants were detected by the presence of a 22 bp deletion in exon 5, Figure 1. To observe this variant, 5 µL of PCR products of KIR2DS4 were additionally electrophoresed for 1 h using 3% agarose gel at 100 Volts and visualized as described previously. The band of the deleted variant was 197 bp while the full-length variant was 219 bp [9].



(**A**)

(**B**)

Figure 1. (**A**) A representational picture of gel electrophoresis using 2% agarose gel showing the presence of all 16 KIR genes tested at their expected sizes (M: DNA marker 100 bp), DRB1 was used as a positive control. (**B**) Gel electrophoresis of KIR2DS4 for some samples showing a full-length (F) at 219 bp, and 22 bp deletion (D) at 197 bp using 3% agarose gel. Heterozygous samples contain both bands (H).

2.5. Histological Assessment of Inflammatory Cells Infiltration in HCC Tumors

Tumor tissues were available from 47 of the 83 HCC subjects for assessment of inflammatory infiltrate. Intratumor inflammatory cellular infiltration was assessed using routine hematoxylin and eosin (H & E) stained sections of liver tissues according to a published protocol [11]. Significant lymphoplasmacytic infiltration was defined as dense multifocal or diffuse infiltration in two or more fields under low power magnification (4× objective lens). Tumor samples were classified into those with significant lymphocytic infiltration and those without significant infiltration. Perinecrotic areas were excluded from the assessment of inflammatory cellular infiltration.

2.6. Statistical Data Analysis

The statistical analysis was performed using SPSS software version 20. In the cases where 25% or more cells had expected count less than 5, the *p*-Value was calculated using Fisher's exact significant test. Pearson Chi square approximate test was used in cases where less than 25% of cells had an expected count less than 5. The *p*-Value of <0.05 was statistically significant, and the odds ratio (OR) was calculated in case of significant difference. The KIR genotypes were characterized based on their frequencies using www.allelefrequencies.net (accessed on 15 May 2023).

3. Results

3.1. Frequencies of KIR Genes and Genotypes in the Studied Populations

The international database for KIR genotypes (www.allelefrequencies.net, accessed on 15 May 2023) was used to identify KIR genotypes in HCC, chronic HCV, and healthy control groups in the present study. Genotype 1 (G1), the only AA haplotype identified in our cohort, was the most frequent genotype observed in the three tested groups. Its frequency, however, was much lower in HCC patients (7.2%) compared to chronic HCV (27.0%) (Table 2). After conducting multivariate regression analysis including age and gender, the KIR AA haplotype was found to be an independent risk factor of HCC development (Table 2).

Table 2. Univariate and Multivariate Regression Analysis of KIR AA haplotype.

	Univariate Analysis		**Multivariate Analysis ***	
	***p*-Value**	**OR (95% CI)**	**Adjusted *p*-Value**	**Adjusted OR (95% CI)**
KIR AA haplotype vs. KIR Bx haplotypes	Between HCC and healthy controls			
	0.003	0.24 (0.09–0.62)	<0.001	0.12 (0.04–0.39)
	Between HCC and HCV groups			
	0.001	0.21 (0.08–0.54)	0.03	0.26 (0.08–0.88)

* multivariate analysis included age and gender adjustment.

In addition, the frequency of genotype 6 (G6) which contains all the KIR genes including the activating ones was significantly high in the HCC patients (19.3%) compared to chronic HCV (8.0%) ($p = 0.02$, OR = 2.7 [1.11–6.79]) and healthy controls (6.7%) ($p = 0.006$, OR = 3.31 [1.35–8.16]).

The frequency of G73 was significantly higher in chronic HCV subjects compared to the healthy control group (6%, $p = 0.04$, OR = 7.53 [0.89–63.65]). Analysis revealed no significant difference among patients and controls regarding the frequency of deleted KIR2DS4 (ID) or the full-length gene. The combined full-length KIR2DS4/ID was significantly higher in the chronic HCV group (29%) compared to HCC (10.8%, $p = 0.002$), but it did not reach statistical significance compared to the healthy subjects (20.17%) (Table 3).

Within samples of control subjects, one sample showed a different genotype characterized by the absence of only three genes (KIR2DS1-3) while the other KIR genes were present. This finding was confirmed by repeated typing for that sample. This genotype is not present in the international database for KIR genotypes (www.allelefrequencies.net, assessed on 15 May 2023).

The frequency of each KIR gene was calculated for the controls, chronic HCV patients with no tumor, and for the HCC cohorts. KIR3DL2, KIR3DL3, KIR2DL4, and KIR3DP1 were identified in all samples of each group while the frequency of the remaining genes ranged from 33% to 100%. The frequency of KIR2DL2 was significantly higher in HCC and chronic HCV patients compared to healthy control subjects ($p = 0.01$) but no significant difference was detected between chronic HCV and HCC patients. In addition, KIR genes 2DL2 and 2DS2 were significantly higher in HCC patients (66/83, 79.51%; 58/83, 69.88%, respectively) compared to healthy control subjects (68/120, 65.7%; 67/120, 55.8%, $p = 0.01$

and 0.04, respectively). Activating KIR genes 2DS1 and 3DS1 were significantly higher in HCC patients (48/83, 57.83%; 45/83, 54.22%, respectively) compared to the chronic HCV patients (36/100, 36%; 34/100, 34%, respectively) (p = 0.01). Inhibitory KIR gene 2DL5 and activating gene 2DS5 were significantly higher in HCC patients (66/83, 79.51%; 42/83, 50.60%, respectively) compared to both chronic HCV patients (63/100, 66%; 33/100, 33%, respectively, p = 0.01), and the healthy control subjects (80/120, 66.6%; 44/120, 36.6%, respectively, p = 0.04) (Table 4).

Table 3. Frequencies of KIR genotypes in the studied populations.

Genotype ID	The Study Groups			*p*-Values			
	HCC (n = 83)	**Chronic HCV (n = 100)**	**Healthy Control (n = 119)**	**P1 HCC vs. HCV**	**P2 HCC vs. Healthy Control**	**P3 HCV vs. Healthy Control**	**P4 among the Three Groups**
1	**6 (7.2)**	27 (27.0)	29 (24.4)	**0.0005**	**0.001**	0.65	**0.001**
6	16 (19.3)	8 (8.0)	8 (6.7)	**0.02**	**0.006**	0.79	**0.01**
5	10 (12.0)	20 (20.0)	14 (11.8)	0.14	0.95	0.09	0.16
2	8 (9.6)	7 (7.0)	11 (9.2)	0.51	0.92	0.54	0.77
4	7 (8.4)	7 (7.0)	8 (6.7)	0.71	0.64	0.93	0.89
71	5 (6.0)	5 (5.0)	6 (5.0)	0.76	0.76	0.98	0.94
19	4 (4.8)	3 (3.0)	1 (0.8)	0.52	0.73	0.33	0.21
7	4 (4.8)	2 (2.0)	6 (5.0)	0.28	0.94	0.29	0.46
73	2 (2.4)	6 (6.0)	1 (0.8)	0.23	0.36	**0.04**	0.07
3	2 (2.4)	4 (4.0)	7 (5.9)	0.53	0.23	0.57	0.48
9	2 (2.4)	1 (1.0)	2 (1.7)	0.45	0.71	0.99	0.75
90	2 (2.4)	1 (1.0)	1 (0.8)	0.45	0.36	1	0.59
93	2 (2.4)	0	0	0.2	0.16	1	NA
81	2 (2.4)	0	1 (0.8)	0.2	0.36	1	NA
18	1 (1.2)	2 (2.0)	0	0.67	0.41	0.2	NA
8	1 (1.2)	0	2 (1.7)	0.2	0.78	0.5	NA
80	1 (1.2)	0	0	0.2	0.41	1	NA
28	1 (1.2)	1 (1.0)	0	0.89	0.2	0.45	NA
64	1 (1.2)	1 (1.0)	0	0.89	0.2	0.45	NA
228	1 (1.2)	1 (1.0)	0	0.89	0.2	0.45	NA
106	1 (1.2)	0	0	0.2	0.2	1	NA
167	1 (1.2)	0	0	0.2	0.2	1	NA
294	1 (1.2)	0	0	0.2	0.2	1	NA
30	1 (1.2)	0	0	0.2	0.2	1	NA
21	1 (1.2)	0	1 (0.8)	0.2	0.79	0.99	NA
69	0	1 (1.0)	2 (1.7)	1	0.51	0.99	NA
70	0	1 (1.0)	2 (1.7)	1	0.51	1	NA
11	0	1 (1.0)	2 (1.7)	1	0.51	0.99	NA

Table 3. *Cont.*

Genotype ID	The Study Groups			*p*-Values			
	HCC (*n* = 83)	**Chronic HCV (*n* = 100)**	**Healthy Control (*n* = 119)**	**P1 HCC vs. HCV**	**P2 HCC vs. Healthy Control**	**P3 HCV vs. Healthy Control**	**P4 among the Three Groups**
175	0	1 (1.0)	0	1	1	0.45	NA
27	0	0	2 (1.7)	1	0.51	0.5	NA
12	0	0	2 (1.7)	1	0.51	0.5	NA
382	0	0	1 (0.8)	1	1	1	NA
331	0	0	1 (0.8)	1	1	1	NA
159	0	0	1 (0.8)	1	1	1	NA
14	0	0	1 (0.8)	1	1	1	NA
20	0	0	1 (0.8)	1	1	1	NA
72	0	0	1 (0.8)	1	1	1	NA
13	0	0	1 (0.8)	1	1	1	NA
35	0	0	1 (0.8)	1	1	1	NA
75	0	0	1 (0.8)	1	1	1	NA
118	0	0	1 (0.8)	1	1	1	NA
68	0	0	1 (0.8)	1	1	1	NA
KIR AA haplotypes	**6 (7.2)**	27 (27.0)	29 (24.2)	**0.0005**	**0.001**	0.65	**0.001**
KIR Bx haplotypes	77 (92.8)	73 (73.0)	91 (75.8)	**0.0005**	**0.001**	0.63	**0.001**
2DS4 full length	29 (34.9)	14 (14.0)	18 (15.13)	**0.0008**	**0.001**	0.82	**0.0004**
2DS4 variant (22 bp deletion)	38 (45.7)	55 (55.0)	68 (57.14)	0.21	0.11	0.75	0.25
2DS4 Heterozygous	9 (10.8)	29 (29.0)	24 (20.17)	**0.002**	0.07	0.12	**0.01**
2DS4 Absence	7 (8.4)	2 (2.0)	9 (7.56)	**0.04**	0.82	0.06	0.11

Absolute numbers represent frequency. The numbers in parentheses represent percentages. ***p***-Values are indicated as P1: HCC vs. chronic HCV, P2: HCC vs. Healthy control, P3: Chronic HCV vs. Healthy control, and P4: Among the three groups. Bolded values represent statistically significant changes. One different genotype characterized by absence of only three activating genes (KIR2DS1-3) is found in healthy subjects that is not present in KIR genotypes international database (www.allelefrequencies.net, accessed on 15 May 2023).

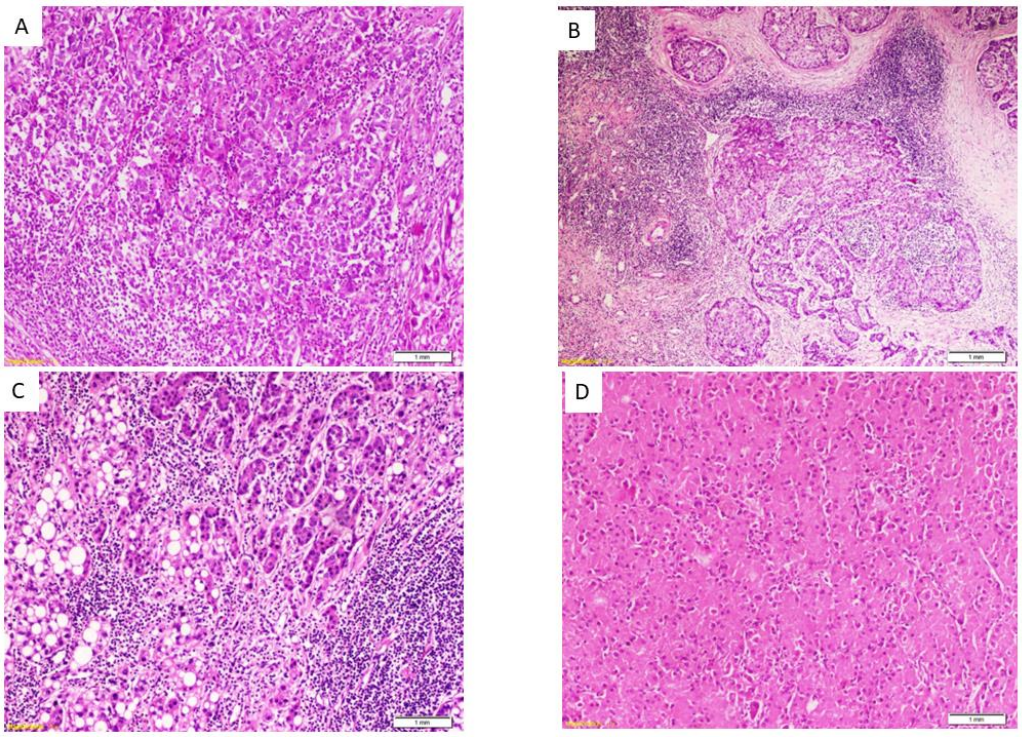
3.2. Correlation between the Degree of Inflammatory Infiltration in the Tumor and KIR Genes

Tissue samples were available from 47 HCC tumors. A total of 21 tumors exhibited a significant inflammatory cells infiltration and 26 showed no or minimal inflammatory infiltrations in their tumors (Figure 2). Although there was no statistical significance due to the limitation of the number of HCC with available tissue samples in this study, the frequency of G4 was higher (14.28%) in the HCC tumors with strong inflammatory infiltration compared with the tumors with no or weak infiltration (3.85%), while the frequency of G6 was higher (30.77%) in tumors with no or weak infiltration compared to those with strong infiltration (9.52%) (Table 5).

Table 4. Frequencies of KIR genes in the studied populations.

KIR Gene	HCC (n = 83)	Chronic HCV (n = 100)	Healthy Control (n = 120)	P1 HCC vs. HCV	P2 HCC vs. Healthy Control	P3 HCV vs. Healthy Control	P4 among the Three Groups
2DL1	82 (98.8)	100 (100.0)	119 (99.1)	1	1	1	1
2DL2	66 (79.51)	64 (64.0)	68 (56.7)	0.18	**0.01**	0.33	**0.03**
2DL3	70 (84.34%)	86 (86.0)	109 (90.8)	0.75	0.15	0.26	0.33
3DL1	79 (95.18)	98 (98.0)	112 (93.3)	0.28	0.58	0.18	0.25
3DL2	83 (100.0)	100 (100.0)	120 (100.0)	NA	NA	NA	NA
2DL5	66 (79.51)	63 (63.0)	80 (66.6)	**0.01**	**0.04**	0.57	**0.04**
3DL3	83 (100.0)	100 (100.0)	120 (100.0)	NA	NA	NA	NA
2DS1	48 (57.83)	36 (36.0)	54 (45.0)	**0.01**	0.2	0.22	0.07
2DS2	58 (69.88)	57 (57.0)	67 (55.8)	0.07	**0.04**	0.86	0.09
2DS3	48 (57.83)	46 (46.0)	50 (41.6)	0.11	**0.02**	0.58	0.07
2DS4	76 (91.57)	98 (98.0)	111 (92.5)	**0.04**	0.8	0.06	0.12
2DS5	42 (50.60)	33 (33.0)	44 (36.6)	**0.01**	**0.04**	0.67	**0.03**
3DS1	45 (54.22)	34 (34.0)	53 (44.1)	**0.01**	0.33	0.13	0.06
2DL4	83 (100.0)	100 (100.0)	120 (100.0)	NA	NA	NA	NA
3DP1	83(100.0)	100 (100.0)	120 (100.0)	NA	NA	NA	NA
2DP1	82(98.80)	100 (100.0)	119 (99.1)	1	1	1	1

NA means not applicable. Absolute numbers represent frequency. The numbers in parentheses represent percentages. The p-Values are indicated as P1: HCC vs. chronic HCV, P2: HCC vs. Healthy Control, P3: Chronic HCV vs. Healthy control, and P4: Among the three groups. Bolded values represent statistically significant changes



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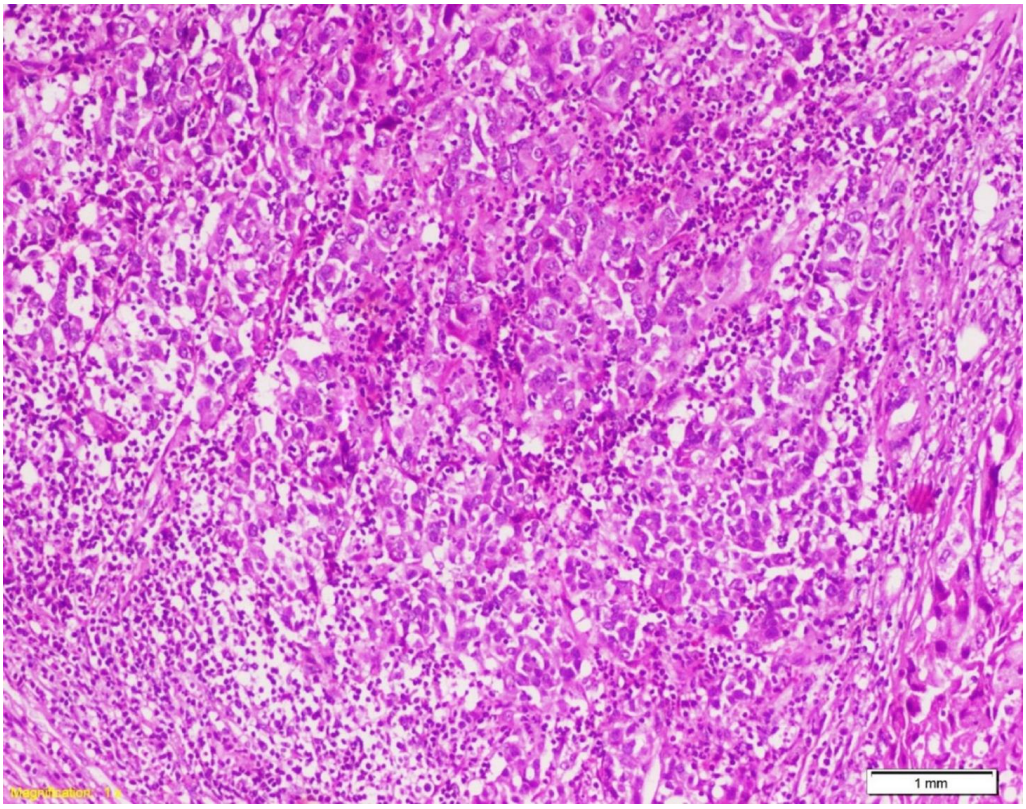

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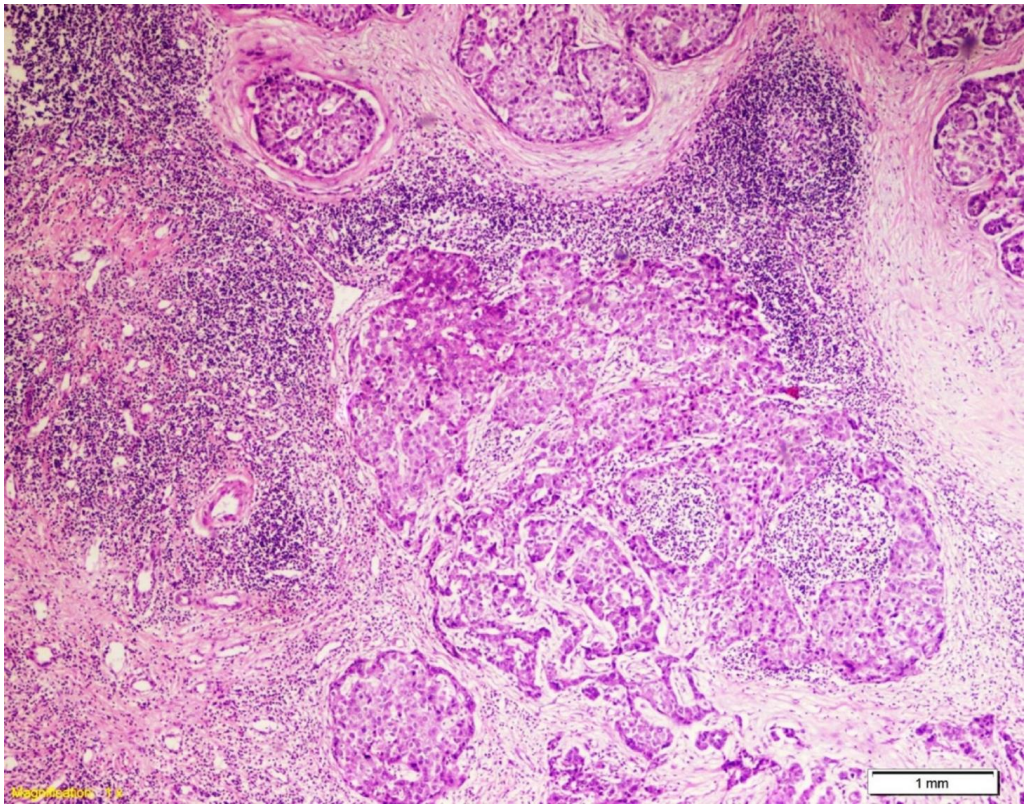

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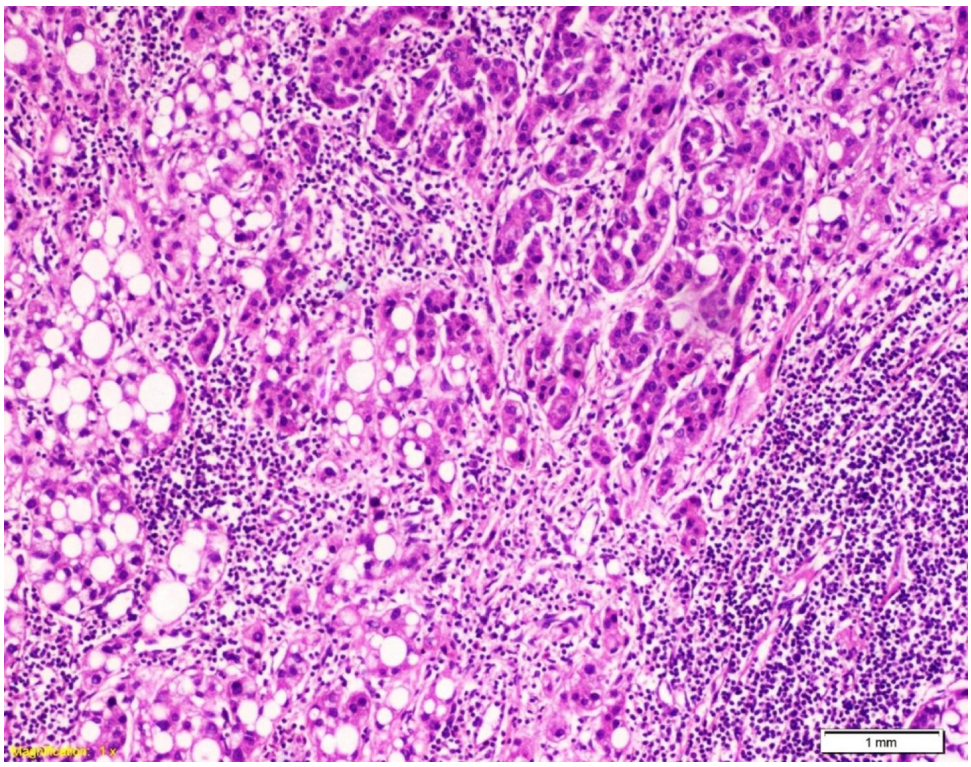
Figure 2. *Cont.*



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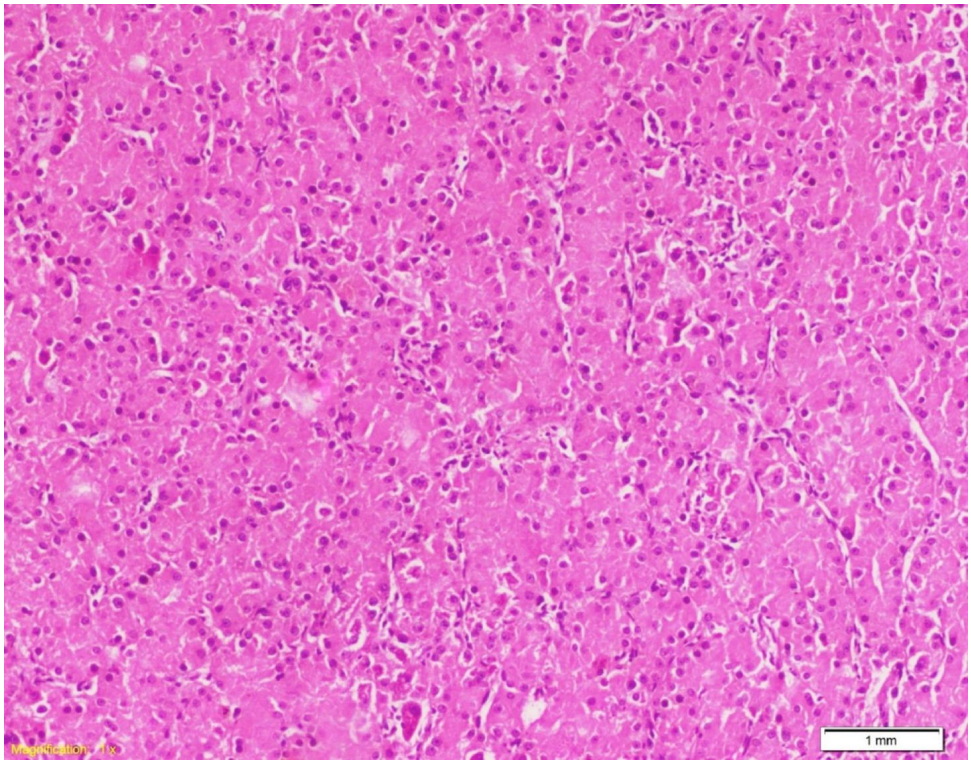

D

Figure 2. Representative examples of patterns of inflammatory cellular infiltration in HCC tumors. (**A**) HCC with significant diffuse lymphoplasmacytic infiltrate (H & E, 200×). (**B**) HCC with significant multifocal lymphoplasmacytic infiltrate (H & E, 40×). (**C**) HCC with significant multifocal lymphoplasmacytic infiltrate (H & E, 200×). (**D**) HCC with mild inflammatory infiltration (H & E, 200×).

Table 5. KIR genotype frequencies and tumor inflammatory infiltrate.

Genotype ID	Frequency in Tumors		*p*-Value
	With Infiltration (n = 21)	Without Infiltration (n = 26)	
1	2 (9.52)	0 (0.00)	0.19
2	1 (4.76)	3 (11.54)	0.4
4	3 (14.28)	1 (3.85)	0.2
5	2 (9.52)	3 (11.54)	0.82
6	2 (9.52)	8 (30.77)	0.07
7	2 (9.52)	1 (3.85)	0.42
8	0 (0.00)	1 (3.85)	1
18	1 (4.76)	0 (0.00)	0.45
19	1 (4.76)	1 (3.85)	0.8
21	0 (0.00)	1 (3.85)	1
28	0 (0.00)	1 (3.85)	1
30	1 (4.76)	0 (0.00)	0.45
71	2 (9.52)	0 (0.00)	0.19
73	0 (0.00)	1 (3.85)	1
81	0 (0.00)	2 (7.69)	0.49
90	0 (0.00)	1 (3.85)	1
93	0 (0.00)	1 (3.85)	1
167	1 (4.76)	0 (0.00)	0.45
294	1 (4.76)	0 (0.00)	0.45

Absolute numbers represent frequency. The numbers in parentheses represent percentages.

4. Discussion

The results of the present work suggest that the G1 KIR genotype, which is an AA haplotype, has a potential role in the prevention of progression of chronic HCV to HCC. No other AA haplotypes were observed in our cohort. This finding is contrary to an earlier report by Littera et al. 2013 who reported a 50% (36/72) frequency of KIR haplotype AA in Italian HCV patients progressing to HCC compared to only 25.6% (40/156) of HCV patients not progressing to HCC [12]. The difference was statistically significant ($p < 0.00001$). HCV genotype difference between the Egyptian and Italian patients is not likely the cause of such discrepancy as the HCV-Genotype 4 patients in the Italian cohort (22% of the total) had the highest overall risk for development of HCC. The most likely explanation of such a discrepancy is the type of treatment the patients received prior to progression to HCC. For the Italian cohort, 63% of the patients were treated by interferon-alpha-based therapies. On the contrary, of our cohort, 60.2% received no prior treatment, 31.3% received direct-acting antiviral (DAA) therapy, and only 8.4% received interferon-based therapies. This suggests that HCV patients with haplotype AA are less likely to progress to HCC with either no treatment or DAA therapies. Our results suggest that KIR allelotype could be used as a prognostic marker for HCV patients on DAA therapy. However, further validation of this observation in an independent cohort with various HCV-genotypes is recommended. The KIR diversity between different populations reported by other groups [13,14] is not a likely explanation as there was no statistically significant difference between the frequency of KIR allelotype in the non-cancer HCV patients in the Italian cohort and the Egyptian cohort (25.6% vs. 27%, $p = 0.8843$).

In reported populations (www.allelefrequencies.net, accessed on 15 May 2023), KIR G1 was observed in 170/171 with overall frequency of 26.6%, which is similar to the frequency

in our control and HCV cohorts. KIR AA haplotype was associated with a protective role against certain studied liver and non-liver diseases. Yindom et al. in 2017 reported that telomeric KIR AA haplotype was associated with reduced risk of e antigenemia and lower viral load, and heterozygosity KIR haplotype (AB) was associated with clearance of hepatitis B virus surface antigen (HBsAg). Moreover, KIR3DS1 was associated with positive HBeAg patients [15]. In line with this, La Nasa et al. in 2016 studied classic Hodgkin lymphoma and found that the KIR AA haplotype alone or in combination with HLA-C1 had a protective effect against progression of the disease [16]. In addition, Barani et al., recently gave evidence for the protective role of KIR AA haplotype and deleted form of KIR2DS4 in head and neck squamous cell carcinoma (HNSCC) in Iranians, and found that KIR2DS1, 2DS5, 3DS1, 2DL5, and CxT4 were the risk factors [17].

The KIR haplotype A has highly variable alleles and constant genes content that enforce immune surveillance against viral infection, while haplotype B contains less allelic polymorphism, and variable genes content have a substantial role in immune-tolerance during gestation [18]. We did not observe a significant difference in the immune cellular infiltrate in tumor tissues with and without the KIR G1 (haplotype AA). However, G4 KIR, which is another genotype with a predominance of inhibitory KIR genes, was significantly associated with strong intra-tumor inflammatory cells infiltration. On the other hand, the frequency of G6 that contained all 16 KIR genes (including all activating genes) was significantly higher in samples of HCC patients compared to the healthy subjects.

These findings suggest that KIR genotypes containing predominantly inhibitory genes may prevent HCC progression via mechanisms that enhance cytotoxicity against tumors. The licensing process may have an influence on this effector function during NK cells' maturation at the developmental stages [19]. Yu et al., 2007 reported that within NK cells' repertoires, the NK cells that have multiple inhibitory KIR receptors for self HLA ligands have a synergistic effect of licensing leading to an increase in their effector capacity [20]. Furthermore, patients with highly licensed NK cells demonstrate a robust immune-surveillance that acts as a potent immune effector against intrahepatic metastasis and de novo carcinogenesis when compared to patients with poorly licensed NK cells [21]. On the other hand, the unlicensed NK cells lack inhibitory receptors to self and their effector function is mediated by an antibody-dependent cellular cytotoxicity (ADCC) mechanism [22]. In this context, previous studies comparing peripheral and liver NK cells reported that the latter exhibit a more potent cytotoxicity against tumors including HCC. This potency is achieved by using a tumor-necrosis factor-related apoptosis-inducing ligand-mediated (TRAIL) mechanism [23]. On the genetic and molecular level, the cumulative risk for HCC recurrence was lower in patients with one or two compound genotypes, than in patients with at least three compound genotypes of KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, or KIR3DL2-A3/11, which are essential to NK cells' licensing [24–26].

De Re et al., 2015 found that the G4 KIR genotype is more frequent in patients with HCV-lymphoproliferative than chronic hepatitis C patients and patients without HCV infection [27]. This genotype is distinct from the G1 KIR genotype by the presence of the KIR2DS2 gene linked to the KIR2DL2 gene through strong disequilibrium linkage (DL). They suggested that the concurrent presence of KIR2DL2 and KIR2DL3 directs HCV progression toward lymphoproliferative disorders. Our results showed that frequency of G4 was higher in HCC patients than the non-HCC groups, while G1 frequency was reduced in the HCC group compared to the non-HCC groups. However, we should note that in our study we observed slightly lower frequencies of KIR3DL1 and KIR2DS4 in HCC patients than in chronic hepatitis C patients while they were higher in the De Re et al. study [27], suggesting a difference in the two populations. Also, Parham in 2004 reported that the absence of KIR2DL2, which has higher affinity for HLA-C1 than KIR2DL3, makes the homozygosity of KIR2DL3/HLA-C1 exert weak inhibition on NK cells, so they will be more easily activated by HCV viral infection [28]. In our study, we found that the frequency of KIR2DL2 was higher in HCC subjects than in both chronic HCV and healthy control groups (Table 4). Although we did not assess the homozygosity for KIR2DL2 and KIR2DL3,

our findings were seemingly consistent with Parham's interpretation suggesting that the KIR2DL2 gene may be associated with HCV resistance and progression to HCC.

Pan et al. demonstrated that HCC incidence in HBV-related HCC patients was associated with the presence of combined full-length KIR2DS4 and 22 bp-deleted forms (ID) along with HLA-C1 and HLA-Bw4I80 [29]. However, in our cohort we did not find significant differences between the combined KIR2DS4/ID in HCC patients and the controls. This may suggest that contrary to HBV infection the combination of KIR2DS4/ID is not associated with HCC in HCV infection.

Kawarabayashi et al., demonstrated that patients with HCV-related cirrhosis and decreased numbers of CD65+NKT cells have a high risk of developing HCC. They concluded that this was due to a reduction in NK cytotoxicity generated by KIR inhibitory signals and an absence or low numbers of activating KIR receptors [30]. In line with this, Littera et al., demonstrated that the frequency of KIR3DS1/HLA-Bw4 combination was much lower in patients with HCV-associated hepatocellular carcinoma than in healthy control subjects, suggested a protective role of KIR3DS1/HAL-Bw4 combination against HCC progression from cirrhotic HCV infection through enhancement of NK cell cytotoxicity [12]. However, we found that the frequency of KIR3DS1 was higher in HCC patients than in the non-HCC groups, and we did not find a difference in frequency of the deleted variant of KIR2DS4 among the studied groups, suggesting a hypothesis that there are host factors other than KIR3DS1 that may have a potential role in controlling HCC progression (Tables 3 and 4).

5. Conclusions

In conclusion, we described the first analysis of Egyptian KIR genotypes from a liver-disease-associated population in the Nile Delta region. The findings of this study revealed that KIR haplotype AA represented by KIR G1 that contains dominantly inhibitory genes has an important role in host defense against HCC progression, in particular in patients treated by non-interferone-based therapies. Further validation study in an independent cohort is recommended.

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Data Availability Statement: Dataset available from the corresponding author at mohamed.abdelrahman@osumc.edu.

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