Chapter 1

Role of Alleles and Genotypes of Polymorphisms of IL-18 (-607 C/A; and -137 C/G), IFN-γ (+874 A/T) and TNF-α (-238 A/G and -308 A/G) and HLA-G Genes in the Susceptibility of Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is a primary malignant tumor of the liver which represents a serious public health problem in the world, corresponding to the fifth more frequent malignant neoplasia among men, the eighth among women, and the third cause of cancer death in the world [1,2]. Seventy to ninety percent of HCC cases occur in patients with cirrhosis or with chronic liver disease, with cell injury followed by regeneration mediated by the immune response playing an important role in hepatocarcinogenesis [3].

In Brazil, a national survey to update HCC epidemiology and clinical profile of patients with HCC (29 centers, with 1,405 patients diagnosed with HCC from 2004 to 2009) showed that the median age at diagnosis was 59 years (1–92 years old; 78% male) and 98% of the patients had cirrhosis (1279/1308), with the hepatitis C virus being the main etiology (54%), followed by hepatitis B virus (16%) and alcohol (14%). In Southeastern and Southern Brazil, hepatitis C virus accounted for over 55% of cases. In the Northeast and North, hepatitis C virus accounted for less than 50%, and hepatitis B virus accounted for 22–25% of cases [4]. In Ribeirão Preto, Southeastern Brazil, the clinical characteristics of 130 patients with HCC attended at the University Hospital of the Faculty of Medicine of Ribeirao Preto, University of São Paulo (HCFMRP-USP) was revised. The mean (± SD) age at the time of HCC diagnosis was 55.6±11.2 years, with 81.5% of them being males. Cirrhosis was present in 89.2% of cases, with 53.4% of

the patients being ChildPugh A; chronic hepatitis B or C without cirrhosis was detected in 3.2%, nonalcoholic steatohepatitis (NASH) in 3.8%, and a normal liver in 3.8% [5].

The human major histocompatibility complex (MHC) represents a set of genes responsible for coding histocompatibility molecules. It is a high density region [3.6 Mb DNA) located on the short arm of chromosome 6, region 6p21.3, which contains more than 200 genes grouped into three classes denoted class I, II and III. Class I genes (classic or class Ia) code for the classic histocompatibility molecules HLA-A, B and C; class II genes code for the histocompatibility molecules HLA-DR, DQ and DP, and class III genes, although included in the MHC, do not code for histocompatibility molecules. Among class I genes, there are also those denoted non-classic or class Ib, which code for the non-classic histocompatibility molecules HLA-E, F and G [6].

HLA-G is a class I non-classic HLA gene consisting of eight exons and seven introns with a stop codon in exon 6 (exon 7 is always absent from mature mRNA and, due to the stop codon in exon 6, exon 8 is not translated), a 5′ promoter region and a 3′ untranslated region (3′UTR) [7,8]. The characteristics of *HLA-G* are: low polymorphism, alternative splicing in the primary transcript that codes for seven protein isoforms, limited distribution in normal tissues, and immunsuppressive properties [9]. Of the seven proteins coded by the *HLA-G* gene, four are linked to the membrane (HLA-G1 to HLA-G4) and three are soluble (HLA-G5 to HLA-G7). The HLA-G1 and HLA-G5 proteins are the main isoforms described in healthy issues such as trophoblast, thymus, cornea, and erythroid and endothelial precursors. On the other hand, the expression of *HLA-G* can be induced in pathological situations such as autoimmune and inflammatory diseases, viral infections, cancer, and transplantation. *HLA-G* exerts inhibitory activity by binding to inhibitory receptors denoted immunoglobulin-like transcript (ILT)-2 and ILT-4, which are expressed by lymphoid and myeloid cells and by myeloid cells only, respectively, and killer immunoglobulin-like (KIR)2DL4 receptor, present only in natural killer cells [9].

Thus far, 47 alleles have been attributed to the *HLA-G* gene, which code for 15 distinct functional proteins with all isoforms (*HLA-G**01:01, *01:02, *01:03, *01:04, *01:06, *01:07, *01:08, *01:09, *01:10, *01:11, *01:12, *01:14, *01:15, *01:16, and *01:17) and truncated (G*01:05N allele) or no proteins at all (G*01:13N allele). Polymorphic sites are observed in the coding and non-coding regions. The promoter and 3′UTR regions of *HLA-G* are highly polymorphic, with the variations in the 3′UTR being associated with the levels of *HLA-G* expression. Two main polymorphic sites were identified in the 3′UTR: a 14bp insertion/deletion, in which the insertion has been associated with reduced levels of HLA-G, and the presence of guanine at position +3142, which increases the affinity of specific microRNAs for *HLA-G* mRNA, reducing the expression of HLA-G [8,10].

The expression of *HLA-G* is frequently detected in malignant tumors of various origins and in some situations has been significantly correlated with tumor size, degree of invasion, metastasis, clinical stages of the disease, and mortality [11,12]. Regarding HCC, few studies evaluating the role of alleles and genotypes of *HLA-G* polymorphism are available in the literature, and evolve different population. Jiang et al, studying a Chinese population showed that the heterozygote and the 14bp II homozygote confer a lower risk of HCC compared with

14bp DD, and that the associations were stronger in the hepatitis B-positive than the hepatitis B-negative population [13] Zhang et al, examining fifteen single-nucleotide polymorphisms (SNPs) in the non-classical class I alleles, found that the SNPs rs17875380, rs41557518, rs114465251, and rs115492845 were associated with susceptibility to chronic hepatitis B infection or HCC, and HLA-F*01:04, HLA-G*01:05N, and HLA-E*01:01 were associated with hepatitis B or hepatitis B with HCC [14].

In general, the inflammatory response mediated by the immune system is beneficial to the host; however, when tissue homeostasis is chronically affected the interactions between innate and adaptive immune responses may be deregulated, culminating with chronic inflammation, excessive tissue remodeling, loss of tissue architecture, apoptosis/necrosis and oxidative stress which, under certain circumstances, may increase the risk of tumor development [15]. The cytokines are responsible for the regulation of growth differentiation and activation of immune cells. The ability to produce cytokines by an individual is influenced by genetic components that have been attributed to molecular mechanisms, including variations in the transcription, translation and secretion pathways [16].

2. Problem statement

Over the last years, regarding HCC, few studies evaluating the role of alleles and genotypes of *HLA-G* polymorphism are available in the literature, and included diverse patient populations. Moreover, previous studies have concentrated on cytokine promoters, encouraged by the identification of a series of SNPs [17]. However, the relation of these polymorphisms with susceptibility to HCC or disease severity has not been clarified.

Application area

Immune response and carcinogenesis

Research course

Alleles and genotypes of cytokines polymorphisms and 14bp of HLA-G gene in HCC patients.

3. Method and patients

We evaluated, in a Brazilian cohort, the association of alleles and genotypes of the 14bp insertion/deletion polymorphism of the *HLA-G* gene, and alleles and genotypes of polymorphisms of genes *IL-18* (-607 C/A; rs1946518 and -137 C/G; rs187238), *IFN-γ* (+874 A/T; rs62559044) and *TNF-α* (-238 A/G; rs361525 and -308 A/G; rs1800629) with susceptibility to HCC and with the type of tumor presentation (infiltrative diffuse, multinodular and uninodular), with nodule size (>10 cm, 5-10 cm and <5 cm), with the Milan criteria (fulfills or does not fulfill), the evaluation of metastasis (present or absent), and the histological classification of the tumor (Edmondson-Steiner Classification) [18,19, 20,21].

This was a retrospective cross-sectional study conducted on 109 patients (89 men) with mean age 55.8±11.4 years for the 14bp insertion/deletion polymorphism of the *HLA-G* gene, and 112 consecutive patients [Mean (±SD) age was 55.6±11.2 years, and 81.5% were males] for the polymorphisms of genes *IL-18* (-607 C/A; rs1946518 and -137 C/G; rs187238), *IFN-γ* (+874 A/T; rs62559044) and *TNF-α* (-238 A/G; rs361525 and -308 A/G; rs1800629) followed up from 2001 to 2009 at the Focal Hepatic Injuries Outpatient Clinic of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCFMRP-USP). The study was approved by the Research Ethics Committee of HCFMRP-USP. Individuals of both genders who fulfilled the following criteria were included in the study: a) diagnosis of HCC defined by the Barcelona 2000 criteria [22] for the diagnoses performed up to 2006 and according to the recommendations of the American Association for the Study of Liver Disease (AASLD) [23] for the diagnoses performed since 2007; b) with DNA stored in the Biological Sample Bank of the Laboratory of Gastroenterology, Department of Internal Medicine. Exclusion criteria were patients with focal hepatic injuries other than HCC according to the Barcelona 2000 criteria and the recommendations of the AASLD and patients with other concomitant neoplasias.

The evaluation of the severity of HCC was based on: a) tumor presentation (uninodular, multinodular or diffuse infiltrative); b) nodule size (<5 cm, 5-10 cm or >10 cm); c) Milan criteria (fulfills or does not fulfill); d) metastasis (present or absent); e) histological classification according to Edmondson & Steiner, 1954 (grades I, II, III or IV) [24].

A total of 202 healthy individuals (56 females and 146 males) with a mean age (±SD) of 33.3±8.3 years and from the same geographic region as the patients studied were used as controls for the evaluation of the frequency of 14bp, *IL-18*, *IFN-γ* and *TNF-α* alleles and genotypes.

For the determination of the 14bp insertion/deletion, IL-18 (-607 C/A; rs1946518 and -137 C/G; rs187238), IFN-γ (+874 A/T; rs62559044) and TNF-α (-238 A/G; rs361525 and -308 A/G; rs1800629) genotypes, we first extracted genomic DNA from peripheral leukocytes by the salting out technique [25]. The HLA-G 14bp insertion/deletion genotypes at exon 8 of the HLA-G locus was analyzed as described: 200ng of genomic DNA were amplified in a 25mL reaction mixture containing 0.20mM dNTP (Invitrogen, Carlsbad, CA), 0.2mM of each primer, 0.5U Taq DNA polymerase (Invitrogen, Carlsbad, CA), 1.5mM MgCl2 and a 1x PCR buffer (0.2M Tris–HCl, pH 8.5; 0.5M KCl). After an initial denaturation step at 94°C for 5 minutes, samples were submitted to 30 additional cycles at 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 1 minute, with a final extension cycle at 72°C for 7 minutes with 5′-TGTGAAA-CAGCTGCCCTGTG-3′ as the forward primer and 5′-AAGGAATGCAGTTCAGCATGA-3′ as the reverse primer [26,27]. After DNA amplification by PCR, the reaction products were submitted to 10% polyacrylamide gel electrophoresis under non-denaturing conditions followed by silver impregnation. The presence of 345 bp fragments corresponded to the deletion allele, while the 359 bp fragment corresponded to the 14bp insertion allele.

The alleles and genotypes of *IL-18* (-607 C/A; rs1946518 and -137 C/G; rs187238), *IFN-γ* (+874 A/T; rs62559044) and *TNF-α* (-238 A/G; rs361525 and -308 A/G; rs1800629) polymorphisms were analyzed by the polymerase chain reaction using allele-specific primers (PCR-SSP) (Table 1). For SNP *IL-18* −607, a generic reverse primer 5′-TAACCTCATTCAGG

SNP	Primer	Sequence (5′-3′)
IL-18 -607 C/A	*IL-18* 607.2	TAACCTCATTCAGGACTTCC
	IL-18 607C	GTTGCAGAAAGTGTAAAAATTATTAC
	IL-18 607A	GTTGCAGAAAGTGTAAAAATTATTAA
	HGBA.S	CGGTATTTGGAGGTCAGCAC
	HGBA.A	CCCACCACCAAGACCTACTT
IL-18 -137 C/G	*IL-18* 137.2	AGGAGGGCAAAATGCACTGG
	IL-18 137C	CCCCAACTTTTACGGAAGAAAAC
	IL-18 137G	CCCCAACTTTTACGGAAGAAAAG
	HGBA.S	CGGTATTTGGAGGTCAGCAC
	HGBA.A	CCCACCACCAAGACCTACTT
IFN-γ +874 A/T	*IFN-γ* 874	TCAACAAAGCTGATACTCCA
	IFN-γ 874 T	TTCTTACAACACAAAATCAAATCT
	IFN-γ 874 A	TTCTTACAACACAAAATCAAATCA
	GH 1	GCCTTCCCAACCATTCCCTTA
	GH 2	TCACGGATTTCTGTTGTGTTTC
TNF-α -238 A/G	TNF 238 UP	AGGCAATAGGTTTTGAGGGCCAT
	TNFAS238G	CCCCATCCTCCCTGCTCC
	TNFAS238A	TCCCCATCCTCCCTGCTCT
	HGBA.S	CGGTATTTGGAGGTCAGCAC
	HGBA.A	CCCACCACCAAGACCTACTT
TNF-α -308 A/G	TNFAA 308.2	CAGCGGAAAACTTCCTTGGT
	TNFAS 308G	ATAGGTTTTGAGGGGCATGG
	TNFAS 308A	ATAGGTTTTGAGGGGCATGA
	HGBA.S	CGGTATTTGGAGGTCAGCAC
	HGBA.A	CCCACCACCAAGACCTACTT

IL-18: interleukin-18; *IFN-γ*: interferon-gamma; *TNF-α:* tumor necrosis factor-alpha; HGBA.S and HGBA.A: human hemoglobin; GH1 and GH2: human growth hormone.

Table 1. Primers for the detection of single nucleotide polymorphisms (SNPs) of genes IL-18 (-607 C/A and -137 C/G), IFN-γ (+874 A/T) and TNF-α (-238 A/G and -308 A/G).

ACTTCC-3′ and two allele-specific forward primers (5′-GT TGCAGAAAGTGTAAAAAT-TATTAC-3′ and 5′-GTTGCAG AAAGTGTAAAAATTATTAA-3′) were used to amplify a 196-bp product. For SNP *IL-18* −137, a common reverse primer 5′-AGGAGGGCAAAATG-CACTGG-3′ and two allele-specific forward primers (5′-CCCCAACTTTTACGGAAG AAAAG-3′ and 5′-CCCCAACTTTTACGGAAGAAAAC-3′) were used to amplify a 261-

bp product. An internal positive amplification control was performed using the primers 5′-CG GTATTTGGAGGTCAGCAC-3′ and 5′-CCCACCACCAAGA CCTACTT-3′, which are specific for the human hemoglobin genes. The reactions were performed in a final volume of 10μL containing 200ng of genomic DNA, 3pmol of each primer (the generic one and a specific one), 2pmol of each control primer, 0.25mM dNTP (Pharmacia Biotech, Paris, France), 1.5mM MgCl2, 0.75U of Taq DNA poly- merase (Invitrogen, Carlsbad, CA), and 1× PCR buffer (0.2 M Tris – HCl, pH 8.5, 0.5 M KCl). The cyclingconditions were 3min at 94°C, followed by seven cycles of 20s at 94°C, 40s at 64°C for *IL18* -607 or 60s at 68°C for *IL18* -137 and 40s at 72°C and 25 cycles of 20s at 94°C, 40s at 57°C for *IL18* -607 or 20s at 62°C for *IL18* -137 and 40s at 72°C, and final stage of 5min at 72°C [9]. For the *TNF-α* (-238 and -308) and *IFN-γ* +874 specific amplification, the primers were identical to those previously described [28,29]. All amplification products were visualized using 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by silver staining.

For statistical analysis, the allele and genotype frequencies were calculated by the direct count method in all groups. Adherence of genotypic proportions to Hardy-Weinberg expectations was determined by the exact test of Guo and Thompson [30] using the GENEPOP software v. 4.0.10. The presence of a significant association between polymorphisms of the same gene was evaluated by a likelihood ratio test of probability of linkage disequilibrium using the ARLEQUIN software, v. 3.1 [31]. If a positive association was detected, but the gameteic phase was unknown, the PHASE (v. 2 package) [32] and EM algorithms [33] were used to reconstruct the TNF or IL haplotypes. Allele, genotype and haplotype frequencies were compared by the two-tailed Fisher exact test using the GraphPad InStat 3.06 software, which was also used to estimate the odds ratio (OR) and its 95% confidence interval (95%CI). The level of significance was set at $P<0.05$.

4. Results

Cirrhosis was observed in 89% of the patients, and major underlying causes included: hepatitis C (35%), alcohol plus hepatitis C (25%), alcohol (18%), hepatitis B (9%), alcohol plus hepatitis B (4%), alcohol plus hepatitis C plus hepatitis B (1%), hereditary hemochromatosis (1%), non-alcoholic steatohepatitis (1%), autoimmune hepatitis (1%) and cryptogenic cirrhosis (5%). Four percent of the patients had no underlying liver disease, 4% non-alcoholic steatohepatitis and 3% chronic hepatitis without cirrhosis (two hepatitis B and one hepatitis C). Fifty-four percent (59/109) of the patients met the Milan criteria for liver transplantation [34]. Metastasis search was performed in 88% of cases (17% had metastasis and 93% did not). Tumors <5cm, 5-10cm and >10 cm were found in 57%, 18% and 8% respectively. Diffuse infiltrative HCC totaled 15% of the cases. Histological evaluation of HCC was performed in 39% (42/109) of subjects with 62% presenting Edmondson-Steiner I or II and 38% III or IV.

4.1. 14bp insertion/deletion polymorphism

Genotype frequencies of patient and control groups were in accordance to Hardy-Weinberg Equilibrium. The 14bp*D allele was more frequent in cases of HCC than in controls (0.6514 vs. 0.5619; *P*=0.0326), conferring an OR=1.46 (95%CI=1.04-2.05). Evaluation of genotype frequency (genotypes 14pb DD, DI and II) did not show significant difference between the groups studied (*P*=0.0871; OR=1.54; 95%CI=0.96-2.48; *P*=0.7182; OR=0.89; 95%CI=0.56-1.44; and *P*=0.1343; OR=0.60; 95%CI=0.32-1.12, respectively). However, the 14bp DD genotype was marginally more frequent among individuals with HCC than among controls, with 10.3% difference (0.4495 vs. 0.3465, respectively) (*P*=0.0871; OR=1.54; 95%CI=0.96-2.48). Patients were stratified according to the characteristics of HCC (type of tumor presentation, nodule size, Milan criteria, presence of metastasis and Edmondson-Steiner classification), and no significant differences were detected between groups regarding the 14bp insertion/deletion allele frequencies (14bp*D and 14bp*I alleles) or genotype frequencies (14bp DD, DI and II genotypes). Table 2 shows the frequency of the 14bp insertion/deletion polymorphism and Table 3 shows the results of the statistical analyses.

Samples	**14bp alleles**		**14bp genotypes**		
I	D	II	DI	DD	
Groups	n(frequency)	n(frequency)	n(frequency)	n(frequency)	n(frequency)
Control	177 [0.4381]	227 [0.5619]	45 [0.2228]	87 [0.4307]	70 [0.3465]
HCC	76 [0.3486]	142 [0.6514]	16 [0.1468]	44 [0.4037]	49 [0.4495]
Tumor presentation					
Diffuse	7 [0.2500]	21 [0.7500]	1 [0.0714]	5 [0.3571]	8 [0.5714]
Multinodular	14 [0.3684]	24 [0.6316]	13 [0.1579]	9 [0.4211]	6 [0.4211]
Uninodular	55 [0.3667]	95 [0.6333]	12 [0.1600]	31 [0.4133]	32 [0.4767]
Nodule size					
>10cm	6 [0.3333]	12 [0.6667]	1 [0.1111]	4 [0.4444]	4 [0.4444]
5-10cm	11 [0.2750]	29 [0.7250]	1 [0.0500]	9 [0.4500]	10 [0.5000]
<5cm	51 [0.4113]	73 [0.5887]	13 [0.2097]	25 [0.4032]	24 [0.3871]
Metastasis					
Present	4 [0.2857]	10 [0.7143]	0 [0.0000]	4 [0.5714]	3 [0.4286]
Absent	63 [0.3539]	115 [0.6461]	13 [0.1461]	37 [0.4157]	39 [0.4382]
Milan criteria					
Yes	46 [0.3898]	72 [0.6102]	11 [0.1864]	24 [0.4068]	24 [0.4068]
No	30 [0.3000]	70 [0.7000]	5 [0.1000]	20 [0.4000]	25 [0.5000]
Edmondson					
I-II	24 [0.4615]	28 [0.5385]	5 [0.1923]	14 [0.5385]	7 [0.2692]
III-IV	16 [0.5000]	16 [0.5000]	4 [0.2500]	8 [0.5000]	4 [0.2500]

HLA-G: H*uman Leukocyte Antigen-G*; bp: base pairs; HCC: hepatocellular carcinoma; I: insertion; D:deletion

Table 2. Distribution of the *HLA-G* 14bp insertion/deletion allele and genotype frequencies.

Comparisons	14bp alleles		14bp genotypes		
I	D	II	DI	DD	
Groups					
HCC *vs.* control	0.0326	0.0326a	0.1343	0.7182	0.0871b
Tumor presentation					
Diffuse *vs.* multinodular	0.4238	0.4238	0.6197	1,0000	0.4905
Diffuse *vs.* uninodular	0.2839	0.2839	0.6830	0.7738	0.3863
Multinodular *vs.* uninodular	1.0000	1.0000	1.0000	1.0000	1.0000
Nodule size					
>10 cm *vs.* 5-10 cm	0.7577	0.7577	0.5320	1.0000	1.0000
>10 cm *vs.* <5 cm	0.6132	0.6132	0.6769	1.0000	0.7318
5-10 cm *vs.* <5 cm	0.1372	0.1372	0.1698	0.7963	0.4382
Milan criteria					
Yes *vs.* no	0.1995	0.1995	0.2794	1.0000	0.3421
Metastasis					
Present *vs.* Absent	0.7739	0.7739	0.5883	0.4556	1.0000
Edmondson-Steiner					
I-II *vs.* III-IV	0.8232	0.8232	0.7109	1.0000	1.0000

HLA-G: *Human Leukocyte Antigen-G*; bp: base pairs; HCC: hepatocellular carcinoma; I: insertion; D:deletion

[a] Odds ratio= 1.46 [95% confidence interval: 1.04-2.05]

[b] Odds ratio= 1.54 [95% confidence interval: 0.96-2.48]

Table 3. Probability values obtained by means of two-tailed Fisher exact test in the comparisons of HLA-G 14bp insertion/deletion allele and genotype frequencies between different groups.

4.2. IL-18 (-607 C/A; rs1946518 and -137 C/G; rs187238), IFN-γ (+874 A/T; rs62559044) and TNF-α (-238 A/G; rs361525 and -308 A/G; rs1800629) polymorphism

The genotypification of the polymorphisms of the genes *IL-18* (-607 C/A and -137 C/G), *IFN-γ* (+874 A/T) and *TNF-α* (-238 A/G and -308 A/G) was performed in 112 patients with HCC and in 202 healthy controls. The genotype distribution of the two groups adhered to the theoretical proportions of Hardy-Weinberg equilibrium. Significant associations were detected between HCC and the following alleles (Table 4): *IL-18* -607*A (*P*=0.0235; OR=1.48; 95%CI=1.06-2.08); *TNF-α* -238*A (*P*=0.0025; OR=2.12; 95%CI=1.32-3.40), and *TNF-α* -308*A (*P*=0.0351; OR=1.82; 95%CI=1.07-3.08). When the genotypes were evaluated (Table 5), the following associations with HCC were detected: *IL-18* -607 AA (*P*=0.0048; OR=3.03; 95%CI=1.40-6.55); *TNF-α* -238 GA (*P*=0.0011; OR=2.44; 95%CI=1.45-4.12); and *TNF-α* -308 GA (*P*=0.0031; OR=2.51; 95%CI=1.39-4.51). Alleles and genotypes from *IL-18* -137G/C and *IFNγ* +874T/A were not

associated with susceptibility to HCC. The inference of haplotypes was performed for the polymorphisms of *IL-18* and *TNF-α*. Haplotypes -607A/-137G IL-18 and TNF-α -308G/-238A -308A/-238G were more frequent in patients with HCC compared with the control group (P = 0.0180, OR = 1.69, 95% CI 1.10 to 2.59, P = 0.0036, OR = 2.06, 95% CI 1,28-3.31 and P = 0.0480, OR = 1.75; 95% CI = 1.03 to 2.97, respectively). On the other hand, TNF-α -308G/-238G haplotype was more frequent in the group of healthy subjects (P = 0.0001, OR = 0.46, 95% CI 0.32 to 0.68), providing protection against HCC.

SNP	**Allele frequency**					
	allele	HCC n (frequency)	Control n (frequency)	P	OR	95%CI
IL-18 -137C/G	C	62 [0.2768]	120 [0.2970]	0.6464	0.90	0.63-1.30
	G	162 [0.7232]	284 [0.7030]	0.6464	1.10	0.77-1.59
IL-18 -607A/C	A	92 [0.4107]	129 [0.3193]	0.0235	1.48	1.06-2.08
	C	132 [0.5893]	275 [0.6807]	0.0235	0.67	0.48-0.94
IFN-γ +874A/T	A	130 [0.5856]	240 [0.5941]	0.8652	0.96	0.69-1.35
	T	92 [0.4144]	164 [0.4059]	0.8652	1.03	0.74-1.44
TNF-α -238A/G	A	41 [0.1847]	39 [0.0965]	0.0025	2.12	1.32-3.40
	G	181 [0.8153]	365 [0.9035]	0.0025	0.47	0.29-0.76
TNF-α -308A/G	A	30 [0.1351]	32 [0.0792]	0.0351	1.82	1.07-3.08
	G	192 [0.8649]	372 [0.9208]	0.0351	0.55	0.32-0.93

Table 4. Distribution of the allele frequencies of polymorphisms of the *IL-18* (-607 C/A and -137 C/G), *IFN-γ* (+874 A/T), and *TNF-α* (-238 A/G e -308 A/G) genes among patients with hepatocellular carcinoma (HCC) and healthy controls.

When the -607C/A SNP of *IL-18* was evaluated in terms of the different presentations of the HCC, the frequencies of the -607*C and -607*A alleles between individuals with multinodular and uninodular HCC, showed that the -607*C allele confers significant susceptibility to multinodular lesions (*P*=0.0289; OR=2.4; 95%CI = 1.09-5.28). Evaluation of the genotype frequency in SNP -607C/A of *IL-18* revealed that genotype 607CC was significantly more frequent in cases of multinodular HCC compared to uninodular tumors (*P*=0.0284; OR=3.5; 95%CI=1.24-9.86). On the other hand, genotype +874AT was found to be more frequent among patients with infiltrative diffuse HCC compared to uninodular HCC (*P*=0.0443; OR=3.6; 95%CI=1.04-12.47), thus conferring greater susceptibility to the diffuse tumor.

SNP	Genotype frequency					
	genotype	HCC n (frequency)	Control n (frequency)	P	OR	95%CI
IL-18 -137C/G	CG	48 [0.4286]	84 [0.4185]	0.9051	1.05	0.66-1.68
	CC	7 [0.0625]	18 [0.0891]	0.5157	0.68	0.27-0.68
	GG	57 [0.5089]	100 [0.4950]	0.9063	1.06	0.67-1.68
IL-18 -607A/C	CA	56 [0.5000]	105 [0.5198]	0.8138	0.92	0.58-1.47
	AA	18 [0.1607]	12 [0.0594]	0.0048	3.03	1.40-6.55
	CC	38 [0.3393]	85 [0.4208]	0.1845	0.71	0.44-1.14
IFN-γ +874A/T	AT	50 [0.4505]	82 [0.4059]	0.4741	1.20	0.75-1.91
	AA	40 [0.3604]	79 [0.3911]	0.6276	0.88	0.54-1.42
	TT	21 [0.1892]	41 [0.2030]	0.8823	0.92	0.91-1.65
TNF-α -238A/G	GA	41 [0.3694]	39 [0.1931]	0.0011	2.44	1.45-4.12
	AA	0 [0.0000]	0 [0.0000]	1.0000	1.81	0.03-92.15
	GG	70 [0.6306]	163 [0.8069]	0.0011	0.40	0.24-0.69
TNF-α -308A/G	GA	30 [0.2703]	26 [0.1287]	0.0031	2.51	1.39-4.51
	AA	0 [0.0000]	3 [0.0149]	0.5548	0.25	0.01-4.99
	GG	81 [0.7297]	173 [0.8564]	0.0098	0.45	0.25-0.80

Table 5. Distribution of the genotype frequencies of polymorphisms of the *IL-18* (-607 C/A and -137 C/G), *IFN-γ* (+874 A/T), and *TNF-α* (-238 A/G and -308 A/G) genes among patients with hepatocellular carcinoma (HCC) and healthy controls.

No significant differences in the allele or genotype frequencies of SNPs of *IL-18, IFN-γ* and *TNF-α* were detected between the various tumor sizes, although the *TNF-α* -238*A allele was slightly more frequent in tumors larger than 10cm compared to tumors smaller than 5cm (*P*=0.0889; OR=2.82; 95%CI: 0.94-8.41). Similarly, genotype *TNF-α* -238AG indicated a marginally greater susceptibility to tumors >10cm (*P*=0.0565; OR=4.63; 95%CI=1.05-20.48) than to tumors < 5 cm, whereas genotype *TNF-α* -238GG conferred marginal protection against large lesions (>10 cm vs. <5cm) (*P*=0.0565; OR=0.22; 95%CI=0.05-0.95).

Evaluation of SNP -607C/A of *IL-18* revealed no significant difference in allele and genotype frequencies between patients with HCC with or without metastasis. In contrast, evaluation of SNP -137C/G of *IL-18* revealed that the -137*C allele was more frequent among individuals with metastasis than among individuals with no meatastasis (*P*=0.0240; OR=4.00; 95%CI=1.32-12.14). The frequencies of genotypes -137CC, CG or GG did not differ significantly between patients with and without metastasis, although genotype -137CC tended to confer greater susceptibility to metastasis (*P*=0.0564; OR=8.80; 95%CI=1.29-60.13) and genotype -137GG presented marginal protection against secondary lesions (*P*=0.0548; OR=0.14;

95%CI=0.02-1.21). Regarding genes *IFN-γ* and *TNF-α*, the allele and genotype frequencies did not differ significantly between the groups with and without metastasis.

The 14bp*D allele in gene *HLA-G* was more frequent in cases of HCC compared to control, with the 14bp DD genotype tending to be more frequent among individuals with HCC. The alleles and genotypes of the 14bp insertion/deletion polymorphism of the *HLA-G* were not associated with disease severity.

Chen et al., in a study of 150 individuals of the Chinese Han population, showed that genotypes of the 14bp insertion/deletion polymorphism of *HLA-G* were significantly associated with the expression of soluble HLA-G in plasma in this population [35]. These authors detected a dramatically lower expression of soluble HLA-G in plasma in the presence of the 14 bp I/I genotype than in the presence of the 14 bp I/D (P = 0.004) or D/D (P = 0.003) genotypes. No significant difference in plasma expression of soluble HLA-G was detected between 14 bp I/D and D/D genotypes.

Many mechanisms of tumor escape have been proposed in the literature, some of them local and others systemic. Particularly important among them is the expression of immunomodulatory molecules in the tumoral microbiota, as well as the expression of soluble suppressive factors by the tumoral cells. HLA-G represents one of these immunomodulatory molecules, playing an important role in the mechanisms of immunotolerance by the inhibition of the activity of NK cells, cytotoxic T lymphocytes and antigen-presenting cells [9,36].

The 14bp insertion/deletion polymorphism in exon 8 of *HLA-G* (3′UTR of the transcript) has been associated with the magnitude of protein production through the modulation of the stability of HLA-G mRNA, although the mechanisms involved still need to be elucidated. It has been demonstrated that the *HLA-G* allele containing the insertion polymorphism may suffer an additional splicing stage, so that 92 bp are removed from the primary mRNA. Thus, smaller *HLA-G* transcripts, without the 92 bp, are more stable than the complete mRNA forms. The alternative splicing may also be related to the presence of other polymorphisms in linkage disequilibrium with the 14bp insertion [8,10]. Overall, HLA-G expression may be modulated by many actors including transcription factors (influenced by the 5′ polymorphisms of the promoter region) and the rate of mRNA degradation or translation, highly influenced by polymorphisms observed at 3′UTR [8]. Ferguson et al. recently reported that the risk for invasive cancer of the uterine cervix in a Canadian population was significantly higher in the presence of 14 bp I/I genotype (OR=2.17, 95% CI: 1.10-4.27, P=0.020) as well as homozygous genotypes HLA-G*01:01:02 (OR=3.52, 95% CI: 1.43-8.61, P=0.006) and *01:06 (OR=19.1, 95% CI: 2.29-159, P=0.005) [12]. Similarly, Chen et al. studying the relationship between HLA-G gene polymorphism and the susceptibility of esophageal cancer in Kazakh and Han nationality in Xinjiang, found that the risk of developing esophageal cancer was significantly increased in individuals with 14 bp I/I genotype compared with 14 bp D/D genotype (OR=2.69, 95% CI: 1.30-5.55, P=0.04) in Kazakh population [11].

The tumoral microbiota or even the cells that underwent mutation per se can induce the expression of HLA-G. Studies have demonstrated the expression of HLA-G in various malignant tumors, although with variations in the percentage of lesions expressing the

molecule. In studies involving renal cell carcinoma [37], endometrial adenocarcinoma [38] and gastric cancer [39], at least 30% of the tumors exhibited HLA-G expression.

Regarding hepatic diseases, Souto et al., in a study of 74 liver biopsies of individuals with chronic HBV infection and 10 specimens obtained from previously healthy cadaver liver donors, demonstrated that 77% of the samples of chronic HBV hepatitis presented HLA-G expression in the hepatocytes, as opposed to none of the controls [40]. These authors detected a case of HCC, in which HLA-G expression was not detected in the tumor cells but was detected in adjacent non-tumoral hepatic tissue.

Lin et al. evaluated by immunohistochemistry the expression of HLA-G in 219 HCC and adjacent nontumoral tissue samples. The expression of HLA-G was observed in 50.2% of HCC samples vs. 0% of normal corresponding adjacent tissue. Evaluation of HCC stages showed that HLA-G expression was detected in 37.8%, 41.9% and 71.4% of cases in stages I, II and III, respectively. The data reported by these authors revealed that the expression of HLA-G was strongly correlated with advanced HCC stage and that soluble HLA-G was significantly more elevated in the plasma of patients with HCC compared to healthy controls [41]. Similarly, Cai et al. studied the expression of HLA-G by immunohistochemistry in 173 HCC specimens and observed that HLA-G expression was associated with the prognosis of HCC, especially in the early stages of the disease, with higher HLA-G expression being independently associated with shorter overall survival and greater tumor recurrence after surgical resection [42].

Recently, a study involving 267 patients divided as anti-HBs positive healthy individuals (n=50), chronic HBV carriers (n=45), active hepatitis B (n=46), liver cirrhosis (n=46) and early-stage HCC patients (n=80) showed that serum concentrations of soluble human leukocyte antigen-G (sHLA-G) were significantly higher in the active hepatitis B and HCC groups compared to the other groups ($P<0.05$). Moreover, the concentrations of sHLA-G were higher in the patients with HCC than in those with liver cirrhosis or active hepatitis B, suggesting that serum sHLA-G concentrations may be associated with the different phases of hepatitis B infection. They did not find any association between sHLA-G concentrations and HCC stage, number of tumors, pathologic grade and presence of vascular invasion [43]. Another study, examining fifteen SNPs in the non-classical class I alleles, found that the SNPs rs17875380, rs41557518, rs114465251, and rs115492845 were associated with susceptibility to chronic hepatitis B infection or HCC, and HLA-F*01:04, HLA-G*01:05N, and HLA-E*01:01 were associated with hepatitis B or hepatitis B with HCC, concluding that these polymorphisms may play an important role in immune surveillance of hepatitis B and HCC, possibly leading to immune responses to virus or cancer cells [14].

It has been demonstrated that, in addition to genetic factors, the microbiota, for example, strees inducers, hypoxia and cytokines (interferons, IL-10, TNF-α), influences the expression of HLA-G, so that more studies are needed for a better understanding of the interaction of molecules derived from the tumor and from host factors [9].

In summary, the present findings show that the deletion allele of the *HLA-G* 14bp insertion/deletion polymorphism was more frequent among patients with tumors than among healthy individuals, a fact that may confer greater susceptibility to HCC.

A higher frequency of the *IL-18* -607*A allele and -607AA genotype was found among HCC patients compared to healthy individuals in the present study. Mi et al. analyzed a pool of studies on the association between polymorphisms of the *IL-18* gene and the risk of cancer (cancer of renal cells, of the ovary and breast, nasopharyngeal an cervical cancer, cancer of the esophagus, prostate, lung and stomach, and colorectal cancer) involving approximately 2137 cases and 3117 controls for the -607C/A variant, and 2372 cases and 3476 controls for the -137G/C polymorphism, and they observed that the -607*A and -137*C alleles were associated with an increased global risk of cancer compared to patients with the wild allele. Analysis of the different ethnic groups showed that these polymorphisms were associated with an increased risk of cancer in Asians but not in Europeans or Africans [44]. Regarding the evaluation of SNP -137C/G, in the present study no significant differences in allele or genotype frequencies were detected between patients with HCC and healthy individuals. However, the *IL18* -137G/-607A haplotype was more frequent among individuals with HCC than among control. These results suggest that, even though no association was detected between SNP -137G/C and HCC, the interaction between the two polymorphisms may have been involved in the susceptibility to HCC. Regarding the polymorphism of the *IFN-γ* gene (+874 A/T), no significant difference in allele or genotype frequencies were observed between patients with HCC and healthy controls. Migita et al. studied 236 Japanese patients with chronic HBV infection who were divided into two groups: with (n=48) and without (n=188) HCC, for the evaluation of the association of polymorphisms of the *TNF-α*, *IFN-γ*, *TGF-β1*, *IL-6* and *IL-10* genes with the risk of HCC. When evaluating the SNP +874 of *IFN-γ*, the authors did not detect a statistically significant difference between the two groups, as also observed in the present study [45].

We observed that alleles *TNF-α* -238*A and -308*A confer significant susceptibility to HCC, and that genotypes *TNF-α* -238GA and -308GA and haplotypes *TNF-α* -238A/-308G and -238G/-308A are also more frequent among patients with HCC compared to control. Also in agreement with these results, Akkiz et al. when analyzing 110 patients with HCC and 110 healthy controls from the Turkish population, observed that patients with HCC had a higher frequency of genotype *TNF-α* -308GA and a lower frequency of genotype *TNF-α* -308GG compared to control and after logistic regression, genotype -308GA was found to be associated with risk of HCC. In addition, individuals with the -308*A allele (genotypes -308AA and -308GA) had a 4.75-fold higher chance to develop HCC compared to individuals with the GG genotype [46]. Similarly, Jung et al., studying 227 Korean patients with HCC and 365 controls, detected a higher frequency of allele -238*A among patients with HCC than among healthy controls [47]. On the other hand, Yang et al., did not find statistical difference in *TNF-α* -308G/A alleles or genotypes frequencies between Chinese HCC patients (n=772) and healthy controls (n=852), but they observed that *TNF-α* -863AA genotype may increase the risk of HCC compared with the wild-type *TNF-α* CC [48].

The expression of *TNF-α* is regulated both at the transcriptional and post-transcriptional level and polymorphisms in the promoter region of *TNF-α* have been related to the production of this cytokine [22]. Greater TNF-α production up to five-fold the basal level and induction of mRNA expression have been associated with the *TNF-α* -308*A allele, with elevated serum TNF levels being observed even in heterozygous patients [49]. Many mechanisms of the

cancerigenous activity of TNF-α have been suggested, such as induction of pro-malignant chemokines, metalloproteinases, cell adhesion molecules, angiogenic mediators, reactive oxygen intermediates and inflammatory enzymes. Increased TNF-α levels are correlated with hepatic inflammation, fibrosis and tissue damage [46].

Studies evaluating the association of polymorphisms of *IL-18* with the severity of cancer have been reported. Saenz-Lopez et al. investigated whether the presence of SNPs -137G/C and -607A/C of *IL-18* were associated with size, grade, and TNM Classification of 158 patients with renal cell carcinoma. These authors observed that genotype -607CC was significantly associated with larger tumor size (P=0.001), grade (P=0.030), and T (P=0.001) and M (P=0.012) stage, while genotype -137GG was correlated with larger tumor size (P=0.036), grade (P=0.017), and stage T (P=0.026) [50]. Another authors, studing the association of SNPs -607A/C and -137G/C of *IL-18* with histology of colorectal and gastric cancer (moderately differentiated or undifferentiated vs. well differentiated), noticed that no difference in genotype frequency was detected, although the combination of genotypes -607AA/-137GC was more frequent among patients with less differentiated tumors [51].

Literature evidence has demonstrated that IL-18 is a pleiotropic cytokine that enhances the Th1 or Th2 immune response according to the medium and to genetics. In the presence of IL-12, IL-18 induces IFN-γ secretion by NK and T cells, activating the Th1 response, important for defense against tumor cells. On the other hand, IL-18 can increase tumor growth via increased stimulation of VEGF and of the immune response, and also stimulate solid tumor metastasis [52]. A possible explanation for this fact is the increased Th1 response in the early stages of the cancer which, however, is replaced with Th2 as tumor malignancy worsens with tumor development. Thus, as the tumor develops, *IL-18* polymorphisms, which induce great IL-18 production, may contribute to the promotion of more advanced tumors due to the activation of angiogenesis, differentiation of tumor cells and regulation of stimulators of cell proliferation [53]. To corroborate these data, Tangkijvanich et al., in a study of 70 patients with HCC and 10 healthy controls, observed that serum IL-18 levels were significantly correlated with the presence of vascular invasion and of more advanced tumors according to the Okuda classification. In addition, the survival of patients with high serum levels of IL-18 was worse. Multivariate analysis showed that serum IL-18 levels proved to be a significant and independent prognostic factor regarding survival [54].

Regarding the +874 A/T polymorphism of the *IFN-γ* gene, study involving 100 patients with chronic HCV infection and different degrees of disease severity (chronic hepatitis, n=42; cirrhosis + HCC, n=58) and 103 healthy controls detected that the TT and AT genotypes were significantly more frequent among patients with cirrhosis and HCC. These genotypes were associated with a 2.5 higher risk of progression to more severe forms of hepatic disease. In addition, the +874*T allele was approximately twice more frequent among patients in an advanced stage of hepatitis C than among patients with chronic hepatitis, although multivariable analysis did not show that the +874*T allele was an independent predictive factor of severity [55].

5. Conclusions

Our results suggest that the 14bp-deletion allele in *HLA-G* gene is associated with HCC susceptibility in a Brazilian population, and that the alleles *IL-18* -607*A and *TNF-α* (-238*A and -308*A) may confer susceptibility to HCC, whereas IL-18 -607*C and -137*C alleles may confer susceptibility to multinodular and diffuse HCC, respectively. Furthermore, deletion/deletion genotype was marginally associated with greater risk of HCC. More studies in different populations are needed to confirm these findings.

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