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ASSESSMENT OF THE ROLE OF NON-CLASSICAL HUMAN LEUCOCYTIC ANTIGENS A-G (HLA-G) VARIATIONS ON HYDATID DISEASE

By

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Abstract

In Egypt, cystic echinococcosis (CE) is a public health problem that categorizes patients into susceptible or resistant groups referring to the immune system, particularly human leukocytic antigen (HLA) Class I & II. In the present study, relation between non-classical HLA -G alleles and the CE was achieved in a retrospective case-control study in Ismailia province. The study included 33 diseased and Control groups matching age, sex, and residence. To identify HLA-G alleles, Polymerase Chain Reaction (PCR) was performed. The frequency of non-classical HLA-G alleles indicated a significant difference between the two study groups in only two (0103 and 0105) out of seven. Allele 0103 was absent in 17 out of 33 (54.5%) patients, while it lacked in 2 control persons (6.1%), however the allele 0105 was lacking in 16 out of 33 (48.5%) patients, while it missed in 3 control persons (9.1%). Regarding cyst sizes, < 5 cm and ≥ 5 cm showed significant differences. There were no significant differences in HLA-G distribution concerning the affected organs.

Keywords: Hydatid cyst, HLA-G, Genotyping, Cyst,

Introduction

Echinococcosis/hydatidosis is a zoonotic parasite caused *E. granulosis* with two types hepatic echinococcosis and alveolar echinococcosis of worldwide distribution in the sheep-raising countries (CDC, 2019), including Egypt (Haridy *et al*, 2006; 2008), and Arab Countries such as Kuwait (Abdul Salam and Farah, 1988), Morocco (Azlaf and Dakkak, 2006), Libya (Kassem and Gdoura, 2006), Saudi Arabia (Fahim and Al Salamah, 2007) and Lebanon (Alam *et al*, 2022). Herbivores animals including man are infected by the ingestion of the *E. granulosus* eggs that can survive up to a year in contaminated food, water and/or soil, or by direct contact with carnivores especially dog (Abdelbaset *et al*, 2021). The prolonged coexistence of parasite within host without effective rejection was not explained by immune system impairment host tolerance (Moro and Schantz, 2009). The initial immune response occurs against the oncospheres that penetrate the gastrointestinal mucosa, and the host mounts the immune response against the metacestode (hydatid cyst). Metacestodes have developed highly effective mechanisms for evading host defenses, as membranes and host capsule surrounding to protect *E. granulosus* cyst from immune destruction. Also, other less well-defined ones, such as parasite-derived modulating substances such as an anti-complement factor, may dampen host immune response (Schantz *et al*, 2011). In resistant patients, some hydatid cysts can grow from 1-50mm/year persisting without changed over the years, or they may exhibit spontaneous rupture, colllapse and disappear (Collado-Aliaga *et al*, 2019). This phenomenon was related to the human leucocytic antigens (HLA) as the strength association of a particular disease particularly the HLA antigens was always quantified (Dorak, 2009). Th1 cytokines mediated a granulomatous reaction in different organs such as the liver, lungs, and other tissues by carbohydrate moieties from *E. granulosus*. Parasite uses such moieties to immunosuppress host and spread locally. This mechanism maintained the infection (Zhang *et al*, 2003).

Class I HLA genes subdivide into class Ia, which includes HLA-A, -B, and -C, & class Ib that provided for HLA-E, -F, & -G (Gros *et al*, 2008). HLA-G may display two distin-

ct activities in pathological conditions; so that it could be protective in the autoimmune and inflammatory diseases or could be suppressive of the immune system in the infections or cancers, and might be used as a novel therapeutic target for new liver diseases (Rashidi *et al*, 2023). Al-Ghoury *et al.* (2010) in Yemen reported that susceptibility to CE in patients was significantly associated with the HLA-DR16 allele and resistance to CE was significantly differed with HLA-DR1, DR8, & DR52 alleles

This study aimed to evaluate possible association between HLA-G antigens expression in Egyptian hydatidosis patients.

Patients and Methods

This analytic case-control retrospective study included 29 patients with hydatid disease in all age groups diagnosed from January 2018 to January 2023. From the medical records of the patients, relevant details were recruited including patients' demographics, presentation, location, cysts number & size, treatment, results and complications. Another 29 cross-matched subjects were recruited as a control without hydatidosis. A complete medical history of the patients and the Control were reported. Every one of the subjects in this study wrote consent. Hydatidosis diagnosis was based on clinical manifestations, imaging (X-ray, ultrasonographic CT, and nuclear magnetic resonance), serological tests, and histological examination. Clinical courses (cyst characteristics, response to medication, failure rate, and recurrent) were detected. Patients treated surgically and/or medically (mebendazole and/or albendazole) were included. Cysts size, number, and site were recorded, and described as successful, failed, or recurrence (Doğru *et al*, 2005).

HLA-G genotyping: Peripheral blood samples (3-5ml) were preserved in ethylene diamine tetra acetic acid (EDTA) coated sterile tubes were used for DNA extraction using DNA isolation kit (QIA amp). DNA Blood Mini Kit (QIAamp DNA Mini Kit: For DNA Purification from Lymphocytes).

DNA extraction and purification was done by three steps: a- Lysing whole blood sample using cell lysis buffer, b- Digestion of cells using proteinase K to de-proteinate sample and inactivating free nuclease, c- DNA was purified using a QIA amp & d- Membrane spin columns: After DNA absorbance and bound to QIA amp, membrane was washed to remove contaminants and eluted by buffer to purify DNA and finally adjusted to 30-100ng/µl. HLA-G genotyping. Mixture reaction contained Tag polymerase thermosaquatics DNA molecules and two specific primers per allele complementary to the two ends of purified DNA (Khehra *et al*, 2023). PCR was used to amplify target sequences.

HLA-G allele typing primers: Genotyping of HLA-G allele was done by PCR-SSP (sequence-specific primer). Only HLA-G alleles specifically coding for nonconservative amino acid substitutions were determined. 11 primer mixes distributed in 4 groups (G1-G4), used for HLAG typing and to detect seven HLA-G alleles with the primers. PCR reaction mixtures (Kovats *et al*, 1990) contained 100ng of genomic DNA; PCR buffer (50mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), & 0.001% [w/v] gelatin); 200-lM each of dATP, dCTP, dGTP, & dTTP; 1 lM of sequence-specific primers; 0.5 lM control primers; and 0.25U of AmpliTaq (Sigma, Bioscience) diluted 1:10 in 13 PCR buffer. Amplification with a GenAmp PCR system 9700 (Applied Biosystems) and carried out for 10 cycles at two temperatures (10 sec at 94^{o}C & 60 sec at 65^{o}C), followed by 20 cycles at different temperatures (30 sec of denaturation at 94^{o}C, 50 sec of annealing at 61^{o}C, & 30 sec of extension at 72^{o}C). in 3% agarose, the amplified products were explored by gel electrophoresis at 150 V for approximately 10min. A specific primer pair for a limited region of the human b-globin gene (present in all DNA samples) was used for patients and control persons of amplification in each PCR reaction.

Positive control included primers of Beta5 50-GCTGCTATCACTTAGACCTCA-30 &

Beta3 50-CTTGTCACAGTGCAGCTCAC-30 produced a fragment of 592 bp. PCR water was added without DNA template.) PCR products were run on 8% non-denaturing polyacrylamide gel electrophoresis, and ethidium bromide stained (Hviid *et al*, 2002).

Statistical analysis: Data of different allele frequencies with the CE infection outcome were correlated statistically using odds ratio and Z test, Chi-square (X2) test with yates' correction was applied for continuity. if $P \leq 0.05$ it was consider significant.

Ethical approval: The study protocol was approval the Ethical Committee of Faculty of Medicine, Suez Canal University, ethical committee No 5715, which when with Helsinki Declaration. The study aim was explanted to all participants and a written consent was taken from each one.

Results

In the present study, patients included 19 (57.6%) females and 14(42.4%) males. Four (12.2%) of them were < 18 years, and 87.8% (29/33) were aged ≥18. Patients 66.7% (22/33) live in rural Suez Canal zone, areas and 33.4% live in urban. A total of 60.6% (20/33) were agricultural workers and 39.4% (13/33) others. Controls matched patients in sex, age, residence, and occupation.

Non-classical HLA-G alleles showed a significant difference between groups in only two (0103 & 0105) out of seven. Allele 0103 was absent in 17/33 patients (54.5%), while it was absent in 2 persons (6.1%) of the control. The allele 0105 was lacked in 16 out of 33 patients (48.5%), while it showed in 3 persons (9.1%) of control.

All patients were diagnosed radiologically with a single cyst/organ/patient, were initially treated medically, and a surgical incision with histopathological confirmation. Cyst sizes < 5cm & ≥ 5cm, with significant differences, but without significant differences in HLA-G distribution of affected organs.

Details were given in tables (1, 2, 3, & 4) and figures (1 & 2)

Table 1: Primer mixes used for HLAG typing used to detect HLA-G alleles

Primer mixes	Nucleotide sequences of primers	Codons Location	PCR (bp)	HLA-G allele-specific amplification patterns by mixes						
				0101	0102	0103	0104	0105	0106	0107
G2–5'	5'-TCCATGAGGTATTTCAGCGC-3	2-10								
G2–3' 31A	5 -CGAACCGCACGAACTGCGT-3'	31-37	100	+	+		+	+	+	+
G2–3' 31T	5'- CGAACCGCACGAACTGCGA-3'	31-37	100			+				
G2–3' 54G	5' -AATACTCCGGCCCCTCCC-3'	54-60	170		+					
G2–3' 54A	5' -CAATACTCCGGCCCCTCCT-3'	54-60	171	+		+	+	+	+	+
G3–5'	5'-CACACCCTCCAGTGGATGAT-3'	93-99								
G3–3' 110A	5' -ATACTGTTCATACCCGCGGAT-3'	110-116	72				+			+
G3–3' 110C	5' -TACTGTTCATACCCGCGGAG-3'	110-116	71	+	+	+		+	+	
G3–3'130CA	5' -GCGGTCCAGGAGCGCAGT-3'	129-135	127					+		
G3–3'130CC	5' –CGGTCCAGGAGCGCAGG-3'	129-135	127	+	+	+	+		+	+
G4–5'	5' -CCACCACCCTGTCTTTGACT-3'	190-197								
G4–3' 258T	5' -CTCATGCTGCACATGGCACA-3	258-264	223						+	
G4–3' 258C	5' -TCATGCTGCACATGGCACG-3' '	258-264	222	+	+	+	+	+		+
G in 1–5'	5' -TCGGGCGGGTCTCAACCT-3'	3'end intron 1								
G2–3' 239T	5' -CGCGGCCGGGCCGGA-3'	13-18	85							+

Table 2: The HLA-G allele-specific amplification patterns of hydatid cyst patients and controls

Groups / Alleles		Control		Patients		Statistical analysis			
		No	%	No	%	Chi X2 &Yates p	Odds ratio	Z statistic	Significance
0101	Present	23	69.7	22	66.7	1.0	1.1500	0.264	P = 0.7916
	Absence	10	30.3	11	33.3				
0102	Present	21	70	20	60.1	1.0	1.1375	0.254	P = 0.7997
	Absence	12	30	13	39.9				
0103	Present	31	93.9	16	48.5	.0001	16.4688	3.465	P = 0.0005
	Absence	2	6.1	17	51.5				
0104	Present	22	66.7	21	63.6	1.0	0.8750	0.258	P = 0.7962
	Absence	11	33.3	12	36.4				
0105	Present	30	90.9	17	51.5	.001105	9.4118	3.209	P = 0.0013
	Absence	3	9.1	16	48.5				
0106	Present	23	69.7	22	66.7	1.0	1.1500	0.264	P = 0.7916
	Absence	10	30.3	11	33.3				
0107	Present	14	42.4	15	45.5	1.0	1.1310	0.248	P = 0.8042
	Absence	19	57.6	18	54.5				

Table 3: Distribution of HLA-G concerning cyst's size

Size / Alleles	< 5 cm		≥ 5 cm		Total		Significance
	No	%	No	%	No	%	
0103	5	15.2	12	36.3	17	51.5	P= 0.0238
0105	11	33.3	5	15.2	16	48.5	
Total	16	48.5	17	51.5	33	100	

Table 4: Distribution of HLA-G concerning affected site

Site / alleles	Liver		Lung		Musculoskeletal		Reproductive		Others		Total		Significance
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
0103	2	6.1	3	9.1	5	15.1	2	6.1	5	15.1	17	51.5	P= 0.799
0105	3	9.1	2	6.1	3	9.1	4	12.1	4	12. 1	16	48.5	
Total	5	15.2	5	15.2	8	24.2	6	18.2	9	27.2	33	100	

Discussion

In the present study, demographic data showed that hydatidosis patients were 19 (57.6%) females and 14 (42.4%) males. This agreed with Heikal and El-Lessy (2021), they found that females were infected (58.06%) than males (41.94 %). the estrogens may have an inhibitory effect on the infection level, but testosterone with little effect (Al-Barwari *et al.*, 1991). In contrast, Salma *et al.* (2014) in Gharbia Governorate reported that hydatidosis was significantly higher in males than in females

In the present study, four patients (12.2%) were < 18 years old, and 87.8% (29/33) were aged ≥18. This agreed with Heikal and El-Lessy (2021), they reported (90.4%) of hydatid cysts was in adults above 20 years old. But, Hassan *et al.* (1996), who reported 11% of Egyptian school children suffered from hepatomegaly and liver hydatidosis. This might be attributed to the chronicity and prolonged prepatent period of hydatid cyst (Asghari *et al*, 2013). Besides, the present study showed that 22/33 (66.7%) of patients lived in rural areas, and 33.4% lived in urban ones Salama *et al.* (2014) in Gharbia Governorate reported that residents of rural areas were more susceptible to hydatid cyst infection than urban ones. Also, Abdel-Moein and Hamza (2016) added that Egyptians in suburban areas were farmers with IHAT 11.9% positive to hydatidosis infection. UNICEF (2023) reported that the Egyptian population live in rural areas (57%) than urban ones (43%). But, Nunnari *et al.* (2012) in Italy found that residence in rural areas might be with increased contact with stray dogs, but Ullah *et al.* (2022) in Pakistan reported that one of the risk factors for hydatidosis was pet dogs. Moreover, the present study showed that 60.6% (20/33) of patients were agricultural workers and 39.4% (13/33) other jobs. Salama *et al.* (2014) reported that a higher hydatidosis was in farmers and housewives associated with dogs. Also, Heikal and El-Lessy (2021) reported that the hydatidosis in the farmers was (32.26%), followed by the housewives (29.03%).

In the present study, the frequency of non-classical HLA-G alleles showed a significant difference between both groups; 2/7 (0103 & 0105), allele 0103 was absent in 17/33 (54.5%) patients compared to 2(6.1%) in control, and 0105 was absent in 16/33 (48.5%) patients compared to 3 (9.1%) in control. This agreed with Al-Marsomy *et al.* (2020), who found that HLA-G alleles were significantly high in hydatidosis patients' pre-surgery (fertile cyst) than post-surgery (sterile cysts). The expression of sHLA-G KIR2DL4 receptors on natural killer cells and some CD8+ T cells may decrease cytotoxic properties or cytokine secretion ability of the lymphocytes (Ristich *et al*, 2005). In hydatid disease, Vuitton *et al.* (2014) found that sHLA-G upregulation was significantly associated with fertile cyst activity than in sterile ones. This may refer to evasion of host immune reaction due to HLA-G expression (Amiot *et al.*, 2015). Also, the primary HLA-G function emerged ability to program cells into immunosuppressive phenotypes (Lin and Yan, 2016) in innate and acquired immune responses (Amiot *et al*, 2014). The-

se functions involved many immune cells that express receptors to HLA-G, such as antigen-presenting cells such as dendritic, T-lymphocytes, monocytes, and Neutral killer cells (Klein and Sato, 2000; Frdric Gros *et al,* 2008). But, non-classical HLA-G in serum and CE evolution was reported (Mariconti *et al,* 2016). Nevertheless, increased sHLA-G levels were reported not only in patients with active hydatidosis, but also in patients with other parasites such as toxoplasmosis, African trypanosomiasis, and American trypanosomiasis (Sabbagh *et al*, 2018).

In the present study, there was significant relationship between alleles and size of hydatid cyst. These were explained by those who showed a negative correlation between sHLA-G and KIR2DL4 (Al-Marsomy *et al.*, 2020). The increase in the expression of this receptor may weaken the host's adaptive immunity by preventing antigen-presenting cells from fulfilling their mission, thereby leading to the evasion of the host's immunity (Amiot *et al,* 2015). Also, HLA-G prevents the phagocytic function of the neutrophils (Baudhuin *et al.,* 2013). HLA-G leads to the suppression of the cytolytic NK cell action; therefore, HLA-G expression in the hydatid cyst cell line plays an essential role in conserving these cells against the attack of NK cells. Thus, uncommon HLA-G expression may contribute to the mechanism of salvation from host immune monitoring to hydatid cysts (Amiot *et al,* 2015). Besides, Abbas *et al.* (2016) reported that the different varieties of *Echinococcus* species may reflect their antigen characterization, wherein complete mitochondrial genome sequencing has identified 10 genotypes.

In the present study, there was no significant relation between sHLA-G distribution, and affected organs. But, high musculoskeletal and other organs prevalence (15.1%) were detected. This agreed with El-Arousy and Ismail (2005), who reported that patients with intracranial or spinal cystic lesions 9 of 14 were hydatidosis seropositive. Also, it agreed with Hussein *et al.* (2012); El-Ghareeb *et al.* (2016) and Abdalla *et al* (2025), they reported that liver and lungs were the most hydatidosis affected organs, and exceptionally others such as kidney, spleen, pancreas, and peritoneal cavity were Also affected. Mazyad *et al.* (1998) reported spinal cord hydatidosis in an Egyptian boy unable to work and Mazyad *et al.* (1999) reported vertebral unilocular hydatidosis in an Egyptian shepherd and his wife. Besides, Gun *et al.* (2017) in Turkey added that the most uncommon hydatidosis site was heart, followed by soft tissue, and gall bladder.

Conclusion

The outcome data showed that sHLA-G detected with higher frequency in CE patients and could be considered a genetic marker for hydatidosis pathogenicity.

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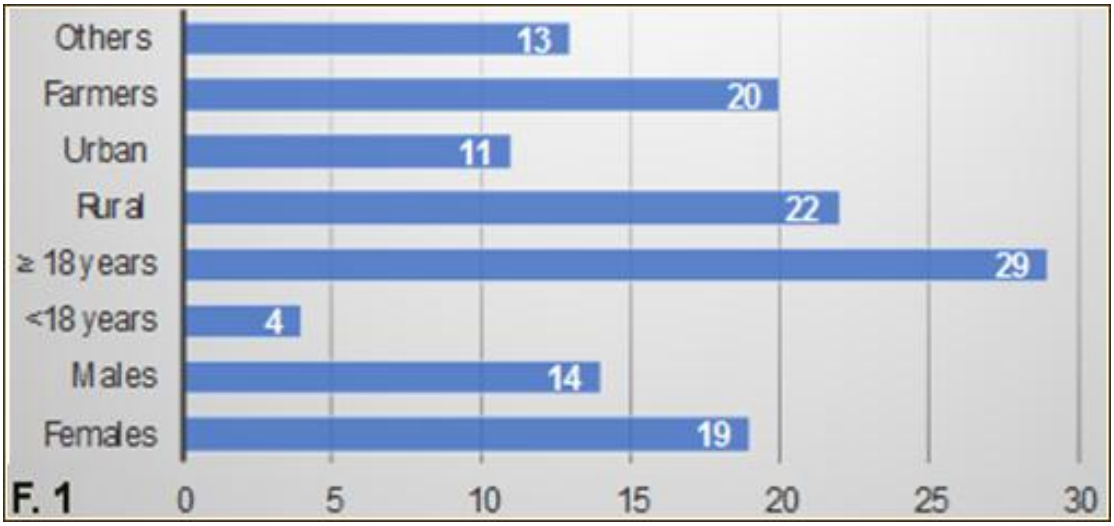
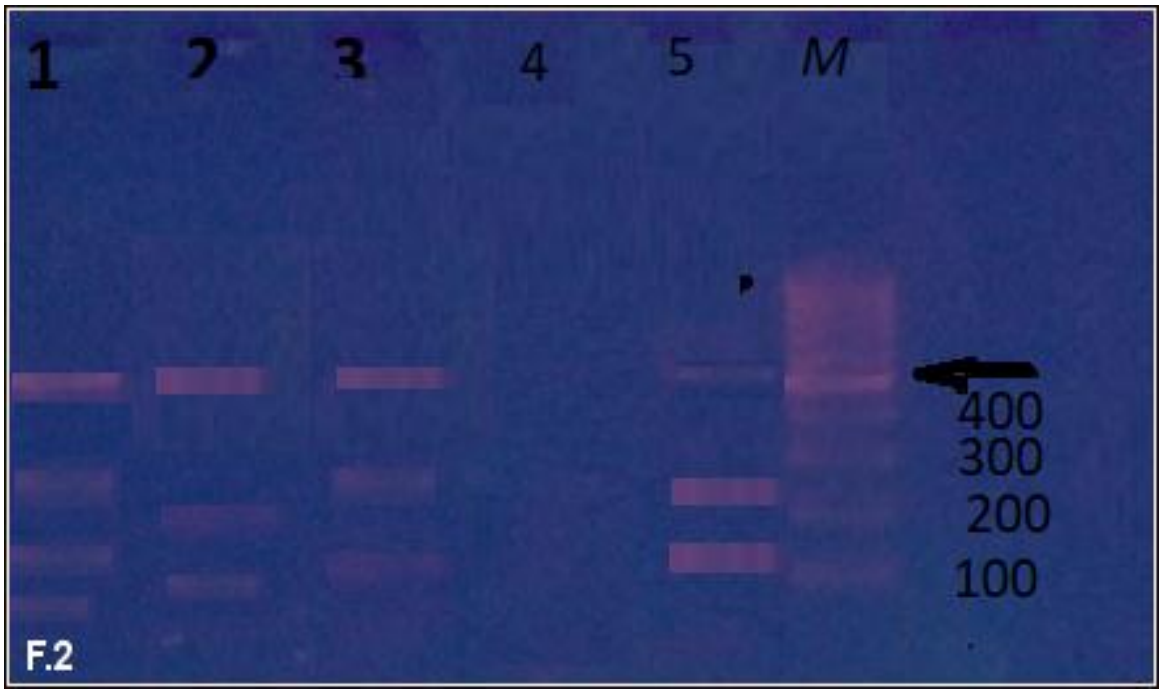
Explanation of figures

Fig. 1: Demographic data of hydatidosis patients.
Fig. 2: PCR products of some samples; M= a ladder 100bp and sample 4 =negative control. Positive control at 592bp showed in each sample. Sample 1 showed three bands (223bp, 100pb & 85pb). Sample 2 showed two bands (170bp &100pb). Sample 3 showed two bands (223 &* 170bp). Sample 5 showed two bands (223 & 170bp).


Others
Farmers
Urban
Rural
≥ 18 years
<18 years
Males
Females
13
20
11
22
29
4
14
19
0
5
10
15
20
25
30
F. 1


1
2
3
4
5
M
400
300
200
100
F.2