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Expression of HLA-G in the Skin of Patients with Pemphigus Vulgaris

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ABSTRACT

Studies on HLA-G, a nonpolymorphic antigen of non-classical HLA class I, is of basic and clinical significance. In the present study, the expression of HLA-G proteins in the human skin tissue sections of normal and autoimmune pemphigus vulgaris (PV) individuals were investigated using monoclonal antibodies.

The antibodies recognized both membrane-bound and soluble isoforms of HLA-G. RT-PCR was performed to assess the patterns of HLA-G mRNA transcripts in the epidermal cells of PV and normal subjects.

HLA-G expression could only be detected at transcriptional level in normal skin tissues. However cells derived from PV subjects expressed detectable HLA-G molecules at both transcriptional and translational levels. In addition, the RT-PCR patterns of HLA-G amplification revealed a reduction in HLA-G2 and an increase in HLA-G1 transcripts in epidermal cells of PV patients as compared to normal cells.

These observations further support suggestions in the literature regarding the role of HLA-G in induction of tolerance in autoimmune individuals.

Key words: Autoimmune disease; HLA-G; Pemphigus vulgaris; Skin

INTRODUCTION

Human leukocyte antigen, HLA-G, is a nonclassical HLA class I molecule which differs from its classical counterparts by its gene being less polymorphic, and encoding proteins which are expressed in lower amounts with a restricted tissue distribution.[1] This protein has been detected on human extravillous cytotrophoblast cells, oocytes, early embryos and thymus.[2] Furthermore, the human choriocarcinoma cell line, JEG-3, naturally expresses HLA-G.[1,3] In addition to the membrane-bound forms, soluble forms of this antigen have been identified in plasma and in amniotic fluid.[2,4] HLA-G molecules can inhibit natural killer (NK)cell-mediated cytolysis and protect semi-allogenic

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fetus from maternal uterine NK cells in pregnancy.[5,6] Due to its presence on chorionic fetal endothelial cells, it has been shown to regulate chorionic villous angiogenesis. Furthermore its soluble isoforms may act as specific immunosuppressors during pregnancy.[7]

Expression of HLA-G in certain human tumors implies a way for the tumor cells to escape immunosurveillance.[8,9] HLA-G protein is expressed in a significant number on endometrial adenocarcinomas, in which it is localized to the glandular epithelium. HLA-G may serve as a clinical marker for the preoperative prediction of metastatic endometrial cancer.[10]

The protein has also been described as a tolerogenic antigen of the MHC and a tissue protective molecule in inflammatory responses.[11] A broader immuno-inhibitory action of HLA-G molecules was described more recently in association with autoimmune diseases.[12]

In the present communication, we report on the differences in the expression of HLA-G in normal and autoimmune (pemphigus vulgaris) individuals at both transcriptional and translational levels.

MATERIALS AND METHODS

Production of MAbs

An HLA-G- specific peptide, consisting of 23 amino acids of the following structure[13,14] was synthesized: EEETRNTKAHAQTDRMNLQTLRG (Severn Biotech Ltd., UK).

A terminal cysteine was attached to facilitate conjugation of the peptide using mailimidobenzoic N-hydroxyl succinimide (MBS, Sigma) as a linker. Keyhole limpet hemocyanin (KLH, Sigma) and bovine serum albumin (BSA) were chosen as carriers and the corresponding conjugates were used for immunization and antibody detection respectively.[15] Hapten: MBS and carrier: hapten ratios (mol / mol) were adjusted at 1:2 and 1:50, respectively. Other details have been described previously.[16]

Preparation of Skin Sample

The patients (PV) biopsies were obtained in cooperation with the Department of Dermatology of Imam Khomeini hospital in Tehran. This study was approved by the Institutional Review Board (IRB) of Faculty of Medical Sciences, Tarbiat Modares University. The patients were diagnosed based on clinical and immunofluorescence criteria by consultant dermatologist and were in the early stage of the disease. They had not received any systemic or local therapy. The biopsies were prepared from the parts of the skin including lesions. Normal foreskin of children 1-5 years old was obtained as control skin.

The specimens were transferred into RPMI medium (GIBCO) supplemented with 10% FCS (GIBCO) and other additives including: 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Sigma), 50 µg/ml gentamycin (Sigma) and 2.5 µg/ml fungizone (Bristol-Myers Sqibb).

Immunofluorescence Staining for HLA-G Expression studies

Skin sections (0.5 µm) from five samples of each normal and patient subjects were prepared. The sections were air dried, fixed with acetone and washed with PBS. The primary Mabs of 5E6H7 [16], specific for all isoforms of HLA-G, prepared in our laboratory or 4H84 specific for all isoforms of HLA-G, kindly provided by S. Fisher and M. McMaster, (University of California, San Francisco were incubated for 35 min at room temperature. After washing, FITC-conjugated rabbit anti-mouse Igs (Fab)' 2 was applied (35 minutes, room temperature). Upon repeating the washing step, mounting solution (50 % glycerol in PBS), was applied on the slide sections before fluorescence microscopy.

Conjugation of MAbs with Fluorochrome dyes

The monoclonal antibodies of LP34 (Dako), specific for cytokeratins 5, 6 and 18, and 5E6H7 were conjugated with FITC (fluorescein-5 isothiocyanate, Merck) and Rhodamine-B-isothiocyanate (Merck) respectively.[17] The conjugates were used for double fluorescence staining.

Double Immunofluorescence Studies

Skin sections of normal and patient (pemphigus vulgaris) individuals were prepared as mentioned above. The fluorochrome conjugates of LP34 and 5E6H7 were added simultaneously and the incubation was carried out for 35 min at room temperature. Upon washing, samples were examined under fluorescent microscope.

Isolation of Epidermal Cells for RT-PCR Studies

Freshly isolated epidermal cells were prepared as follows:[18,19]

Skin samples were fragmented with sterile scalper and scissors and tissue fragments were washed in sterile PBS. Dispase II (Roche) was added (4U/ml) and the incubation was carried out at 4º C overnight. The epiderm and derm layers were separated using two fine scalpers. The epidermal layer (5 mm) was immensed into 500 µL Trizol LS reagent (Life Technologies) solution to denature the proteins and preserve the RNA molecules.

Isolation and Culture of Human Foreskin Dermal Fibroblasts

Small pieces of dermal layer from normal skin were isolated by fine scissors and scalpel. The dermal cells were treated with trypsin (0.2 %). They were then washed and cultured in RPMI medium supplemented with 10% FCS and antibiotics. The confluent cells were used as negative control for HLA-G transcription.

Preparation of Extra Villous Cytotrofoblast from Term placenta

Biopsies of term placenta related to extra villous cytotrofoblast region were fragmented into small pieces and introduced to 500 µL Trizol™ LS reagent solution and used as positive control for HLA-G transcription.

Total RNA Isolation

A solution of sodium acetate (50 µL, 3 M, pH: 5.2) was added to 500 µL of epidermal cells lysate in Trizol LS, followed by the addition of 500 µL nuclease-free water saturated phenol. Subsequently, 200 µL of chloroform-isoamylalcohol mixture, adjusted at 24: 1 (vol /vol), was added and the mixture was incubated for 8-10 min at 4º C. After centrifugation (11000 RPM for 6 min), cold isopropanol was added in equal volume to the supernatant and kept at -20º C for one hour according to a previously reported procedure. [20] Upon centrifugation, the pellet was washed with cold ethanol, dissolved in warm distilled water and kept in a freezer (-70º C).

Reverse Transcription

For the first strand cDNA synthesis, the total RNA was denatured at 65º C in distilled water for 8 min. 5 µg of the RNA was added to the reaction mixture and incubated at 38º C or 55 min. The reaction mixture consisted of: 5 µL of 5x buffer (Roche), 1 µL of deoxynucleoside triphosphates (DNTP, 10 mM), 2.5 µL of dithiothreitol (DTT, 100 mM), 1 µL of oligo-dT (0.5 µg/ µL), 0.5 µL of random hexamer (1 µg / µL), 0.5 µL of RNasin (36200 U/µL, Promega), 1 µL of MMLV reverse transcriptase (Roche) and 8.5 µL of distilled water.

PCR Amplification

PCR was carried out with 5 µL of each cDNA sample in a total volume of 20 µL of the reaction mixture containing: 2 µL of the pan-HLA-G primers (5- GGA AGA GGA GAC ACG CAA CA-3˘), (5´ - GGC TGG TCT CTG CAC AAA GAG A-3) or β-actin primers 5 (5- TCG TCG TCG ACA ACG GCT CC-3), 3 (5-GAA GCA TTT GCG GTG GAC GA-3), 2.5 µL of 10x reaction buffer (Roche), 2 µL of MgCL2 (50 mM), 1 µL of dNTP (50 mM), 11 µL of d H2O and 0.5 µL of Taq DNA polymerase (Roche).

cDNA related to β-actin (1.1- kb fragment) was amplified as a control for RNA quality. PCR was run at 94º C for 1 minute, at 58º C for 1.5 minutes, and at 72º C for 2 minutes for 35 cycles, with a final extention of 10 minutes at 72º C. A total of 20 µL of each PCR reaction was analyzed following electrophoresis on a 1.5 % agarose gel and staining with ethidium bromide.

Statistics

Intergroup differences were tested by non-parametric Mann-Whitney test. A *P*-value of less than 0.05 was considered significant.

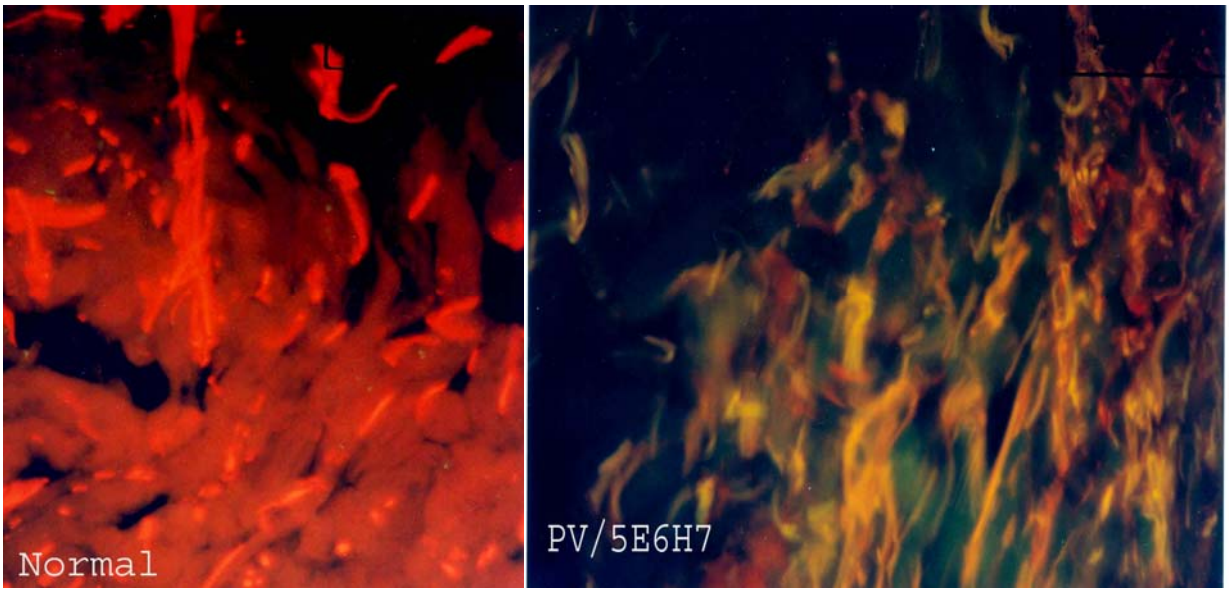
RESULTS

HLA-G protein Expression Studies

Immunofluorescence staining of the normal and patient (PV) skin sections using Mabs 5E6H7 and 4H84 revealed that the HLA-G protein is expressed in four out of five patient samples but in none of the normal controls (Figure 1). To elucidate expression of HLA-G by human epidermal keratinocytes in normal and patient (PV) samples, two fluorochrome conjugates, one specific for human HLA-G and the other for cytokeratins were employed. Double staining with both monoclonal antibodies to keratinocyte specific antigens (cytokeratins) and HLA-G indicated that HLA-G protein is expressed by the keratinocytes of PV individuals (Figure 2).

RT-PCR Studies

β-actin primers were used in RT-PCR studies of the epidermal skin cells to determine the quality of the extracted RNAs and the relative expression of HLA-G.



a b

Figure 1. Immunofluorescence staining used to detect HLA-G in human a) normal and b) patient (PV) skin sections using Mab 5E6H7. No fluorescence labeling (green color) is seen for the normal skin whereas green-yellow color staining of patient's skin shows the reactivity with FITC-conjugate. The type of positive cells is not known in this study. Red color corresponds to Evans blue dye that was employed to neutralize auto fluorescence specificity of the skin tissue.

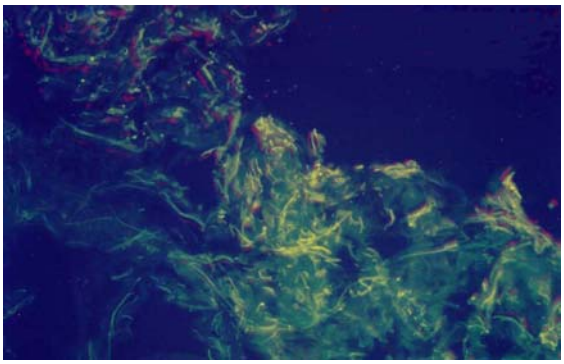


Figure 2. Double Immunofluorescence of PV skin section with anti-HLA-G (red cells) and anti-cytokeratins (green cells). Double-positive cells show yellow color related to simultaneous expression of HLA-G and cytokeratins by skin keratinocytes. Excitation at 568 and 488 nm were used for rhodamine and FITC, respectively. Immunofleorescence image was obtained at approximately 400 × magnification.

The RT-PCR method was simultaneously applied for both normal and patient samples using HLA-G and β-actin primers as described under Materials and Methods. Human term placenta was used as positive control. Using a software program (labworks™) densitometric (O.D.) analysis of each band related to the PCR products was carried out. The ratios of O.D.s related to HLA-G2 (700 bps) and β-actin (1100 bp) indicated a reduction in HLA-G2 transcript in the epidermal cells of patients as compared to the normal cells ($p < 0.05$) (Figure 3).

DISCUSSION

The presence of HLA-G transcripts has been demonstrated in a wide variety of cells in fetal and adult tissues, although the detected levels of mRNA are very low and could merely represent basal transcription.[21] The role of HLA-G in maternal-fetal immune tolerance and protection against natural killer cells cytolysis has been demonstrated.[22]

Recent studies suggest that HLA-G expression is involved to heart and liver-kidney graft acceptance[23,24] and certain autoimmune and inflammatory diseases. Lack of soluble isoforms of HLA-G has been reported in human thyroid follicular cells of graves-basedow.[25] However, HLA-G expression was shown in psoriatic skin sections and not in normal skin controls. [26] In a different study related to rheumatoid arthritis patients, increased levels of HLA-G5 were detected in their synovial fluids.[27]

Organ-specific autoimmune diseases like PV is usually associated with a deficiency in tolerance

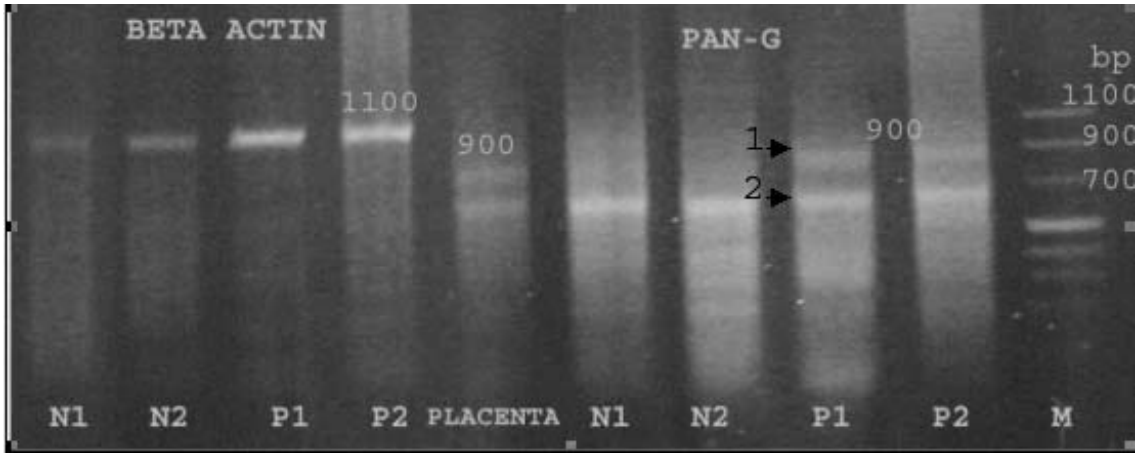


Figure 3. RT-PCR amplification results taken (photographed) with primers of HLA-G and β-actin genes to determine the relative expression of HLA-G. Skin epidermal cells of normal (N) and PV (P) individuals were used. This figure shows that HLA-G1 transcript (900 bps, arrow 1) exists in PV and not in normal samples. Whereas HLA-G2 transcript (700 bps, arrow 2) indicates a decrease in epidermal cells of the patients as compared to the normal cells. A sample derived from term placenta was used as positive control.

induction mechanisms.[28] In the present study, expression of HLA-G in PV was employed as an index of tolerance induction. Based on these findings, it is postulated that the detection of HLA-G in human tissues either in membrane-bound or soluble forms may be useful in distinguishing pathological conditions such as PV. In a different study, Gazit *et al* recently shown that HLA-G expression is associated with PV in jewish patients.[29] Our studies using RT-PCR method have revealed an increase in HLA-G1 and a decrease in HLA-G2 transcripts in PV epidermal cells as compared to normal individuals ($p < 0.05$). These results suggest that different patterns of HLA-G transcription occur in the epidermal cells of normal and PV individuals. Furthermore, HLA-G synthesis is accomplished in most of the PV skin samples and not in normal individuals.

Our suggestion is in line with previous reports related to the role of HLA-G in induction of tolerance. Such an event is important in providing protection of cells or tissues from destruction by natural killer or inflammatory cells. It may also play a final mechanistic role in relation to induction of tolerance in inflammatory circumstances such as autoimmune diseases.

This notion is in line with a recent observation indicating that HLA-G and NK receptors are expressed in psoriatic skin and act to down-regulate the deleterious effects of T-cell infiltrate in this disease.[26] It may also act as an inhibitory factor, like prostaglandin E2, or transforming growth factor-β that are produced from epidermal keratinocytes and contribute to the immunohomeostatic balance of the skin at the end of inflammation.[30] A role of HLA-G in providing repair by diminishing inflammation is also suggested by this mechanism.

In conclusion, it is suggested that the expression of HLA-G gene is regulated at both transcriptional and translational levels with distinct differences in normal and PV individuals. Establishing the mechanism by which HLA-G would modulate inflammatory responses would need further investigations.

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REFERENCES

1. Carosella ED, Moreau P, Le Maoult J, Le Discorde M, Dausset J, Rouas-Freiss N. HLA-G molecules: from maternal-fetal tolerance to tissue acceptance. Adv Immunol 2003; 81:199-252.
2. Kapasi K, Albert SE, Yie S, Zavazava N, Librach CL. HLA-G has a concenteration-dependent effect on the generation of an allo-CTL response. Immunology 2000; 101(2):191-200.
3. Burt D, Johnston D, Rinke de wit T, Vanden Elsen P, Stern PL. Cellular immune recognition of HLA-G-expressing

choriocarcinoma cell line Jeg-3. Int J Cancer Suppl 1991; 6:117-22.
4. Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. J Immunol 1994; 153(12):5516-24.
5. Rouas-Freiss N, Goncalves RM, Menier C, Dausset J, Carosella ED. Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. Proc Natl Acad Sci U S A 1997; 94(21):11520-5.
6. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, and Carosella ED: HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. J Immunol 2001; 166(8):5018-26.
7. Le Bouteiller P, Solier C, Pröll J, Aguerre-Girr M, Fournel S, Lenfant F. Hum Reprod Update 1999; 5(3):223-33.
8. Paul P, Rouas-Freiss N, Khalil-Daher I, Moreau P, Riteau B, Le Gal FA, et al. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. Proc Natl Acad Sci U S A 1998; 95(8):4510-5.
9. Adrian Cabestre F, Moreau P, Riteau B, , Ibrahim EC, Le Danff C, Dausset J, et al. HLA-G expression in human melanoma cells: protection from NK cytolysis. J Reprod Immunol 1999; 43(2):183-93
10. Barrier BF, Kendall BS, Sharpe-Timms KL, Kost ER. Characterization of human leukocyte antigen-G (HLA-G) expression in endometrial adenocarcinoma. Gynecol Oncol 2006; 103(1):25-30.
11. Carosella ED, Moreau P, Aractingi, Rouas-Freiss N. HLA-G: a shield against inflammatory aggression. Trends Immunol 2001; 22(10):553-5.
12. Verbruggen LA, Rebmann V, Demanet C, De Cock S, Grosse-Wilde H. Soluble HLA-G in rheumatoid arthritis. Hum Immunol 2006; 67(8):561-7.
13. McMaster M, Zhou Y, Shorter S, Kapasi K, Geraghty D, Lim KH, et al. HLA-G isoforms produced by placental cytotrophoblasts and found in amniotic fluid are due to unusual glycosylation. J Immunol 1998; 160(12):5922-8.
14. McMaster MT, Librach CL, Zhou Y, Lim KH, Janatpour MJ, DeMars R, et al. Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. J Immunol 1995; 154(8):3771-8.
15. Peeters JM, Hazendonk TG, Beuvery EC, Tesser GI. Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. J Immunol Methods 1989; 120(1):133-43.
16. Yari F, Hosseini AZ, Nemat-Gorgani M, Sareh S, Khorramizadeh MR, Mansouri P, et al. Production and characterization of monoclonal antibodies with specificity for human HLA-G isoforms. Hybrid Hybridomics 2003; 22(5):301-6.
17. Storch Wulf B. Introduction to the basic principles and techniques of immunofluorescence and production of antibodies and labeling with fluorochromes. In: Klein RA, editor. Immunofluorescenece in clinical immunology, A primer and atlas, translated by. Berlin, Germany: Birkhauser, 2000: 1-46.
18. Ulbrecht M, Rehberger B, Strobel I, Messer G, Kind P, Degitz K, et al. HLA-G: expression in human keratinocytes in vitro and in human skin in vivo. Eur J Immunol 1994; 24(1):176-80.
19. Linge C. Establishment and maintenance of normal human keratinocyte cultures. In: GE Jones, Totowa NJ, editors. Methods in molecular medicine, human cell culture protocols. US: Humana press Inc, 1996: 1-8.
20. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162(1):156-9.
21. Onno M, Guillaudeux T, Amiot L, Renard I, Drenou B, Hirel B, et al. The HLA-G gene is expressed at a low mRNA level in different human cells and tissues. Hum Immunol 1994; 41(1):79-86.
22. Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL. HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci U S A 1996; 93(1):161-5.
23. Lila N, Carpentier A, Amrein C, Khalil-Daher I, Dausset J, Carosella ED. Implication of HLA-G molecule in heart-graft acceptance. Lancet 2000; 355(9221):2138.
24. Creput C, Le Friec G, Bahri R, Amiot L, Charpentier B, Carosella E. et al. Detection of HLA-G in serum and graft biopsy associated with fewer acute rejections following combined liver-kidney transplantation: possible implications for monitoring patients. Hum Immunol 2003; 64(11):1033-8.
25. Castro MJ, Morales P, Catálfamo M, Fernández-Soria V, Suárez B, Varela P, et al. Lack of HLA-G soluble isoforms in Graves-Basedow thyrocytes and complete cDNA sequence of the HLA-G*01012 allele. Eur J Immunogenet 1998; 25(4):311-5.
26. Aractingi S, Briand N, Le Danff C, Viguier M, Bachelez H, Michel L, et al. HLA-G and NK receptor are expressed in psoriatic skin: a possible pathway for regulating infiltrating T cells? Am J Pathol 2001; 159(1):71-7.
27. Le Discorde M, Rouas-Freiss, Moreau P, Menier C, McCluskey J, Carosella ED. Functions of human leukocyte antigen-G in health and sickness. Tissue Antigens 2003; 62(4): 273.
28. Shevach EM. Organ autoimmunity. In: Paul WI, editor. Fundamental Immunology. Philadelphia: Lippincott Raven, 1999.
29. Gazit E, Slomov Y, Goldberg I, Brenner S, Loewenthal R. HLA-G is associated with pemphigus vulgaris in Jewish patients. Hum Immunol 2004; 65(1):39-46.
30. Arnola B, Schonrich G, Ferber I, Alferink J, Hammerling GJ. Peripheral T cell tolerance: Distinct levels and multistep mechanisms. Transgen Target Mutagen Immunol 1994:135-47.