Original Article

Evaluation of HLA-G 14bp Ins/Del and +3142 C/G Polymorphisms in Type 1 Diabetes among South Indian Population

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Abstract

Background: Type 1 diabetes (T1D) is a multifactorial autoimmune disease, involving strong genetic components with familial predisposition. Human leukocyte antigen-G (HLA-G) is a non-classical HLA-class I molecule having several immunomodulatory functions. Polymorphisms in *HLA-G* are associated with several autoimmune diseases including T1D. This study aims to evaluate the association of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms with T1D among the South Indian population. **Methods:** The study was performed in a cohort of 123 T1D patients along with their 51 siblings and 126 parents. The association and linkage of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms with T1D were analysed, and transmission disequilibrium test (TDT) was performed. **Results:** Significantly increased frequencies of *HLA-G* 14bp Del/Del genotype (OR = 2.16, p_c = 0.0302) and Del allele (OR = 1.71, p_c = 0.0398) were observed in female patients compared to parents. Higher frequencies of DelDel/GG combined genotype (OR = 4.45, p_c = 0.0049) and Del/G haplotype (OR = 2.91, p_c = 0.0277) were observed in female patients compared to parents. TDT also revealed over-transmission of Del/G haplotype (25T vs 7UT; P = 0.0015) and a strong linkage disequilibrium between the studied polymorphisms. **Conclusion:** This familial study shows the association of *HLA-G* 3′UTR 14bp Ins/Del polymorphism with the risk of T1D among the South Indian population, especially in females.

Keywords: HLA-G, polymorphism, South India, type 1 diabetes

Introduction

Type 1 diabetes (T1D) is a multifactorial autoimmune disease, leading to the destruction of pancreatic β-cells that eventually result in total insulin dependency.[1] This selective destruction of β-cells of pancreatic islets in T1D is due to the complex interaction among the β-cells, the immune system and environmental factors in genetically susceptible individuals.[2] International Diabetes Federation (IDF) has reported that India has the largest number of children and young adults with T1D in the world.[3] Previous studies have demonstrated the complexity of T1D involving various genetic and environmental factors.[2,4] Several T1D-associated loci have been identified through genetic studies and are mapped to allelic variants of known genes.[5]

Human leukocyte antigen-G (HLA-G) is a non-classical HLA-class I molecule with multiple immune-regulatory properties. The HLA-G gene (*HLA-G*) is located on chromosome 6p21.31[6] comprising eight exons and seven introns with 5′ upstream regulatory region (5′URR) and a 3′ untranslated region (3′UTR).[7] It is implicated in the differentiation of antigen-presenting cells and B-cells.[8] It induces the regulatory T-cells,[9] inhibits $CD4^+$ T-cell proliferation,[10] inhibits $CD8^+$ T-cells,[11] and activates NK cells.[12] *HLA-G* expression is seen to be up-regulated in several inflammatory conditions.[13,14] The constitutive expression of *HLA-G* at low levels in the secretory granules of pancreas prevents the activation of

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auto-reactive T-cells, resulting in immune tolerance. This may potentially regulate the immunogenetic ligands present at the sites of insulin exocytosis. The disturbance in this homeostatic state may result in the initiation of inflammation.[15]

HLA-G is a candidate gene for T1D, and its aberrant expression might contribute to T1D susceptibility.[16] The 14-base pair (14bp) insertion/deletion (Ins/Del) polymorphism (rs66554220) and +3142 C/G polymorphism (rs1063320) at 3′UTR of *HLA-G* are among the most widely studied *HLA-G* variants.[17,18] *HLA-G* 14bp Ins/Del polymorphism plays an active role in mRNA stability, affecting the expression of HLA-G protein. Particularly, the 14bp insertion has been associated with higher mRNA instability which consequently lower mRNA production for most membrane-bound and soluble isoforms. Reports have also advocated the strong association of 14bp insertion with low HLA-G production and the 14bp deletion with high expression of *HLA-G*.[17,19] The role of *HLA-G* 14bp Ins/Del polymorphism has been documented in cancers,[20] viral infections[21] and autoimmune diseases.[14,22,23] The 14bp Ins/Del polymorphism is closely associated with the +3142 C/G polymorphism of *HLA-G*.[18] The +3142 C/G polymorphism has been reported to play an important role in the post-transcriptional regulation of *HLA-G*. It is located within the putative binding site for microRNAs containing a guanine residue which enhances the affinity for microRNAs that lead to the down-regulation of the *HLA-G* expression.[17,24] Studies have evidenced the association of *HLA-G* +3142 C/G polymorphism with autoimmune diseases like systemic lupus erythematosus[25] and rheumatoid arthritis.[18]

The association of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms with T1D is inconsistent among various ethnic populations.[16,26,27] Meanwhile, there are no studies describing the association of these polymorphisms among the South Indian population. Henceforth, the present study aims to evaluate the genetic contribution of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms towards the development of T1D among the South Indian population.

Materials and Methods

Study subjects

The recruitment of 123 T1D patients, along with their siblings (n = 51) and parents (n = 126) were done through the Department of Diabetology, Government Rajaji Hospital, Madurai, India. Based on age at diagnosis, the patients were stratified into three groups: Group A, from 0–10 years (n = 32); Group B, from 11–20 years (n = 60) and Group C, >20 years (n = 31). The diagnosis of T1D was carried out by the physicians according to the criteria of American Diabetes Association.[1] Briefly, the diagnosis was based on the clinical and laboratory findings including elevated levels of blood glucose, ketoacidosis with severe symptoms of acute onset at presentation and absolute insulin dependency. The study was approved by the ethics committees of the Government Rajaji Hospital (Reference number: 23339/E4/3/10; Dated: 12th April 2011) and Madurai Kamaraj University (Dated: 24th November 2011). Written informed consent was obtained from all the participating individuals or their guardians for their participation and the use of patients data for research. Moreover, the study was carried out in accordance with the declaration of Helsinki.[28]

Genotyping

Blood samples (3–5mL) were collected from all the individuals, and genomic DNA was extracted by salting out method.[29] The 14bp Ins/Del and +3142 C/G polymorphisms in the 3′UTR of *HLA-G* were genotyped by polymerase chain reaction–sequence-specific primers (PCR-SSP) method.[30] The gel images of 14bp Ins/Del and +3142 C/G polymorphisms are shown in Supplementary Figure 1.

Statistical analysis

The statistical power of the study to determine the significance was found to be more than 90%, between patients and parents and 84%, between patients and siblings. Allele and genotype frequencies were determined by direct counting method and compared between patient groups and siblings/parents by means of Chi-square test, odds ratio (OR) and 95% confidence interval (CI) using *Epi info v7* with Yate's correction. Deviations from Hardy–Weinberg equilibrium (HWE), haplotype frequencies, transmission disequilibrium test (TDT) and linkage disequilibrium (LD) analyses were computationally inferred in patient groups, siblings and parents using *Haploview v4.2*. The *P* value <0.05 was considered statistically significant.

Results

Subject characteristics

The present study comprised 70 male and 53 female T1D patients. The mean age at diagnosis of the T1D patients was 15.5 ± 7.8 years, and the duration of T1D was 8.2 ± 5.9 years. The study included 51 siblings (31 males and 20 females) and 126 parents (48 fathers and 78 mothers). The demographic characteristics of the participating individuals are tabulated in Table 1.

Genotype and allele frequencies

The *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms adhered to HWE (p > 0.05) among patient groups, siblings and parents except for 14bp Ins/Del polymorphism among fathers (p = 0.042). The statistically significant frequency distribution of genotypes, alleles, genetic models, combined genotypes and haplotypes of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms are shown in Table 2. The frequency of Ins/Del genotype was significantly higher among mothers and female siblings compared to female patients. On the other hand, a significantly increased frequency of Del/Del genotype was observed among female patients when compared to mothers.

Subsequently, increased frequency of Ins allele was observed among parents when compared to female patients, meanwhile the Del allele showed a higher frequency among female patients

than parents. However, there were no significant differences in the genotype and allele frequencies of *HLA-G* +3142 C/G polymorphism among the study population.

The recessive model (InsIns+InsDel vs DelDel) of 14bp Ins/Del polymorphism was observed to be significant in female patients when compared to parents, especially mothers (OR = 0.4, p_c = 0.0187). Further, the co-dominant model (InsIns+DelDel vs InsDel) of 14bp Ins/Del polymorphism was found to be significant in female patients when compared to mothers (OR = 2.39, p_c = 0.0274) and female siblings (OR = 4.54, p_c = 0.0123).

Combined genotype and haplotype analyses

The frequency of InsDel/GG combined genotype was observed to be significantly higher in male patients (OR = 3.44, p_c = 0.0322) when compared to fathers. Meanwhile, DelDel/GG combined genotype frequency was significantly higher in patients (OR = 3.5, p_c = 0.0075), group A patients (OR = 4.76, p_c = 0.0107), group B patients (OR = 3.4, p_c = 0.0288) when compared to parents. Similarly, significantly increased frequency of DelDel/GG combined genotype was observed in female patients when compared to parents (OR = 4.45, p_c = 0.0049). However, the combined genotypes InsIns/CC, InsIns/CG and InsDel/CC were not observed in the study population. The haplotype analysis exhibited a significantly increased frequency of Del/G haplotype among female patients (OR = 2.91, p_c = 0.0277) as compared to fathers. The distribution of statistically significant frequencies for *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms between the female T1D patients and parents is depicted in Figure 1.

TDT and LD analyses

TDT was performed with the 39 trio families [Table 3]. Mendelian error was not observed in the study population. The haplotype analysis revealed a significant over-transmission of Del/G haplotype in trio families (25T vs 7UT; P = 0.0015). Also, over-transmission of Del allele and G allele were observed in the single-marker analysis. Further, the pairwise LD analysis of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms among the study cohort [Figure 2] showed a strong LD among the trios (D′ = 1; LOD = 13.28; r^2 = 0.342), parents (D′ = 1; LOD = 15.15; r^2 = 0.343) and fathers (D′ = 1; LOD = 8.33; r^2 = 0.427).

Discussion

Development of T1D involves strong genetic components with familial predisposition.[4] Several genetic loci are involved in the pathogenesis of T1D.[31] The association of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms has been reported in various autoimmune diseases like multiple sclerosis,[13] systemic lupus erythematosus,[22] rheumatoid arthritis.[32] The immunomodulatory HLA-G molecule in pancreatic islets is known to down-regulate the immune responses associated with autoimmunity.[15] Moreover, *HLA-G* polymorphisms have been reported to be associated with T1D.[16,26,27]

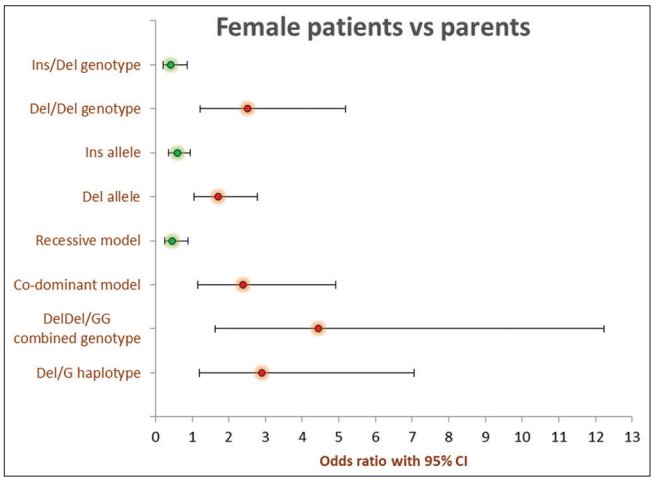

Figure 1: Odds ratio (OR) distribution of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms between the female T1D patients and parents. The ORs <1 (green colour) and >1 (red colour) indicates the association of the polymorphisms with protection and susceptibility to T1D, respectively

Table 1: Demographic characteristics of T1D patients, siblings and parents

Variables	Patients (n=123)	Patients			Siblings (n=51)	Parents (n=126)	P
		Group A (n=32)	Group B (n=60)	Group C (n=31)			
Age, years (mean±SD)	23.6±9.8	15.7±6.7	22±6.5	35±7.4	17.8±9.9	42.6±8.6	0.0003*
							0.0062†
							<0.001‡
Age at Diagnosis, years (mean±SD)	15.5±7.8	6±2.9	15.1±2.6	25.9±4	-	-	<0.001§
Duration of T1D, years (mean±SD)	8.2±5.9	9.7±6.6	6.9±5.5	9.1±5.3	-	-	0.0218‖
							0.0363¶
Gender							
Male (%)	70 (56.9)	18 (56.3)	33 (55)	19 (61.3)	31 (60.8)	48 (38.1)	<0.001**
							0.0428††
Female (%)	53 (43.1)	14 (43.8)	27 (45)	12 (38.7)	20 (39.2	78 (61.9)	<0.001**
							0.0289††

SD: Standard deviation, P values from statistical tests: *t-test between patients and siblings; †t-test between group B patients and siblings; ‡t-test between group C patients and siblings; §t-test within the patient groups; ‖t-test between group A and group B patients; ¶t-test between group B and group C patients; **χ^2 test between patients and siblings; ††χ^2 test between patients and parents

Table 2: Analysis of *HLA-G* 14bp Ins/Del and+3142 C/G polymorphisms

	Female patients (53)	Parents (126)			Fathers (48)			Mothers (78)		
	n (%)	*n* (%)	OR (95% CI)	P_c	*n* (%)	OR (95% CI)	P_c	*n* (%)	OR (95% CI)	P_c
Ins/Del genotype	18 (34)	59 (46.8)	0.58 (0.3-1.14)	0.1552	16 (33.3)	1.03 (0.45-2.35)	1	43 (55.1)	0.42 (0.2-0.86)	**0.0274**
Del/Del genotype	28 (52.8)	43 (34.1)	2.16 (1.13-4.15)	**0.0302**	19 (39.6)	1.71 (0.78-3.77)	0.2572	24 (30.8)	2.52 (1.22-5.19)	**0.0187**
Ins allele	32 (30.2)	107 (42.5)	0.59 (0.36-0.95)	**0.0398**	42 (43.8)	0.56 (0.31-0.99)	0.0641	65 (41.7)	0.61 (0.36-1.02)	0.0787
Del allele	74 (69.8)	145 (57.5)	1.71 (1.05-2.77)	**0.0398**	54 (56.3)	1.8 (1.01-3.21)	0.0641	91 (58.3)	1.65 (0.98-2.79)	0.0787
Recessive model InsIns + InsDel vs DelDel			0.46 (0.24-0.89)	**0.0302**		0.59 (0.27-1.29)	0.2572		0.4 (0.19-0.82)	**0.0187**
Co-dominant model InsIns + DelDel vs InsDel			1.71 (0.88-3.34)	0.1552		0.97 (0.43-2.22)	1		2.39 (1.16-4.92)	**0.0274**
Combined genotype DelDel/GG	11 (20.8)	7 (5.6)	4.45 (1.62-12.23)	**0.0049**	2 (4.2)	6.02 (1.26-28.77)	**0.0286**	5 (6.4)	3.82 (1.24-11.76)	**0.0286**
Del/G	23 (42.5)	40 (31.7)	1.65 (0.85-3.19)	0.1873	10 (20.8)	2.91 (1.2-7.05)	**0.0277**	23 (28.8)	1.83 (0.88-3.8)	0.1469
		Siblings (51)			Male Siblings (31)			Female Siblings (20)		
Ins/Del genotype	18 (34)	24 (47.1)	0.58 (0.26-1.28)	0.2457	10 (32.3)	1.08 (0.42-2.77)	1	14 (70)	0.22 (0.07-0.67)	**0.0123**
Co-dominant model InsIns + DelDel vs InsDel			1.73 (0.78-3.81)	0.2457		0.93 (0.36-2.38)	1		4.54 (1.49-13.8)	**0.0123**
	Male patients (70)	Parents (126)			Fathers (48)			Mothers (78)		
Combined Genotype InsDel/GG	20 (28.6)	26 (20.6)	1.54 (0.78-3.02)	0.28	5 (10.4)	3.44 (1.19-9.94)	**0.0322**	21 (26.9)	1.09 (0.53-2.23)	0.9683
	Parents (126)	Patients (123)			Group A (32)			Group B (60)		
Combined Genotype DelDel/GG	7 (5.6)	21 (17.1)	3.5 (1.43-8.57)	**0.0075**	7 (21.9)	4.76 (1.53-14.78)	**0.0107**	10 (16.7)	3.4 (1.23-9.44)	**0.0288**

Ins: Insertion, Del: Deletion, OR: Odds ratio, CI: Confidence interval, P_c: Yate's corrected *P* value, P_c values highlighted in bold are statistically significant (<0.05)

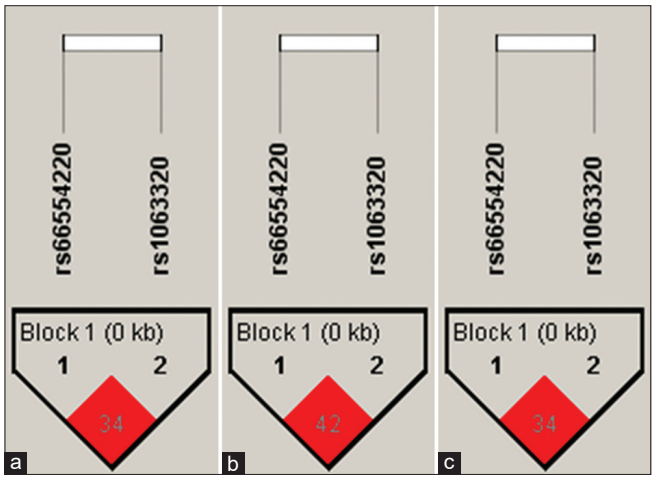

Figure 2: Linkage disequilibrium (LD) analysis of rs66554220 and rs1063320 generated by Haploview v4.2 using r2 and LOD. The number in the box denotes the r2 value between the polymorphisms. D´ values were 1 representing the maximum possible LD. r2: correlation coefficient between the two loci; D´: Measure of normalized linkage disequilibrium; LOD: log of the likelihood odds ratio. (a) Trio families. (b) Fathers. (c) Parents

Table 3: Transmission disequilibrium test for *HLA-G* 14 bp Ins/Del (rs66554220) and +3142 C/G (rs1063320) polymorphisms in 3′ UTR of *HLA-G* in trio families

	T	UT	Chi-square	*P*
Haplotype rs66554220/rs1063320				
Ins/G	14	23	2.189	0.139
Del/G	25	7	10.125	**0.0015**
Del/C	14	23	2.189	0.139
HLA-G 14bp Ins/Del				
Del	23	14	2.189	0.139
HLA-G+3142C/G				
G allele	23	14	2.189	0.139

T: Transmitted, *UT*: Untransmitted, *P* value in bold is statistically significant (<0.05)

Although the association of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms has been reported among several ethnic populations,[18,23,32] the current study evaluates the association of *HLA-G* 14bp Ins/Del and +3142 C/G polymorphisms with T1D among the South Indian population. The outcome of the present study suggests a strong association between the *HLA-G* 14bp Ins/Del polymorphism and T1D, precisely, the Del/Del genotype and Del allele among female patients. Similarly, Del allele and Del/Del genotype corroborate the association with autoimmune diseases such as pemphigus vulgaris[33] and rheumatoid arthritis.[34] The co-dominant model of *HLA-G* 14bp Ins/Del polymorphism reveals the association of this polymorphism with increased risk towards T1D among females. On the other hand, the recessive model of *HLA-G* 14bp Ins/Del polymorphism attributes to a decreased risk of T1D. Likewise, the Ins allele also plays a protective role towards the development of T1D. Moreover, the results expose the significantly increased frequency of Ins/Del genotype among the mothers and female siblings. Similarly, the occurrence of increased frequency of Ins/Del genotype has been reported in controls as compared to patients with juvenile idiopathic arthritis.[34] This notion is supported by similar results of Silva *et al*. on the association of Del allele and Del/Del genotype with susceptibility and Ins allele and Ins/Del genotype with protection towards T1D.[16] Although not statistically significant, the increased frequencies of Ins/Ins genotype (22.6%) and Ins allele (41.9%) among patients with age at diagnosis >20 years could be associated with delayed disease manifestation. Likewise, the Ins allele has been reported to be associated with late-onset T1D in the Greek population.[26]

Studies have demonstrated the association of *HLA-G* +3142 C/G polymorphism with autoimmune diseases like systemic lupus erythematosus,[25] rheumatoid arthritis[18] and T1D.[27] Our results did not show significant association of *HLA-G* +3142 C/G polymorphism with T1D. The discrepancy between the results of the present study and previous reports may be due to differing ethnicity, genetic diversity, sample size and other risk factors. However, in our study, significantly higher frequency of DelDel/GG combined genotype in patients indicates an increased risk towards the development of T1D, especially in younger age groups (≤20 years). Further, the increased frequency of Del/G haplotype suggests the contribution of *HLA-G* +3142 C/G polymorphism in combination with 14bp Ins/Del polymorphism towards T1D development, especially among females. Although similar findings have not been reported in association with T1D, the Del/G haplotype and DelDel/GG combined genotype are shown to be associated with rheumatoid arthritis among the Iranian population.[32]

The results of this study infer that the *HLA-G* 14bp Del allele, homozygous Del/Del genotype, DelDel/GG combined genotype and Del/G haplotype could confer susceptibility to T1D among the South Indian population. On the other hand, the *HLA-G* 14bp Ins allele and heterozygous Ins/Del genotype might confer protection to T1D. Moreover, the combined effect of *HLA-G* 3′UTR 14bp Ins/Del and +3142 C/G polymorphisms might play a significant role in the immunopathogenesis of T1D. Further, it is intriguing to observe the association of *HLA-G* 14bp Ins/Del polymorphism with increased risk of T1D among females, which might be due to several gender-specific factors. The gender-specific involvement of HLA-G polymorphisms may be due to the regulation of HLA-G expression by progesterone through the presence of a progesterone response element at the 5′ upstream region of the gene.[35] In the present study, a significantly high Del/Del genotype and Del allele were observed among females. In agreement with this, the Del/Del genotype and Del allele of HLA-G 14bp Ins/Del polymorphism have been reported to be a risk factor for juvenile idiopathic arthritis, especially in females.[34] In addition, Huxley *et al*. have reported that females have a higher relative risk and mortality rates towards T1D than males.[36]

The results reveal high LD between *HLA-G* 3′UTR 14bp Ins/Del and +3142 C/G polymorphisms. Earlier studies have also reported similar incidence of high LD between both the polymorphisms.[7,18,24] In the present study, TDT of trio families shows that the *HLA-G* 14bp Del allele and +3142 G allele are over-transmitted from parents to T1D patients. Further, the over-transmission of Del/G haplotype confirms the presence of genetic linkage between these alleles. In addition, other polymorphic loci with strong LD that presents within 3′UTR and HLA region might also influence the association of HLA-G with T1D.[7,37]

In conclusion, this could be the first familial study to evaluate the association of *HLA-G* 3′UTR 14bp Ins/Del and +3142 C/G polymorphisms with T1D and the results provide strong evidence for the association of *HLA-G* 3′UTR 14bp Ins/Del polymorphism with the risk of T1D among the South Indian population, especially in females. However, *HLA-G* +3142 C/G polymorphism did not show any association towards the development of T1D. Further analysis of genetic variations in combination with various other factors and HLA-G expression studies could lead to a better understanding of the role of HLA-G in T1D. However, studies with larger sample size and more of complete families are obligatory to establish a definitive association of *HLA-G* 14bp Ins/Del and *HLA-G* +3142 C/G polymorphisms with T1D.

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Conflicts of interest

There are no conflicts of interest.

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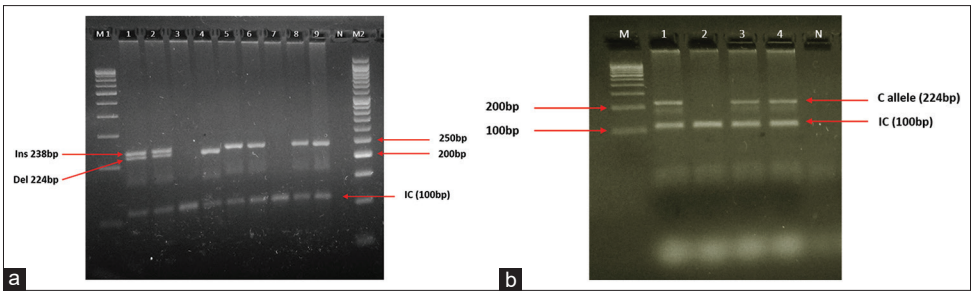

Supplementary Figure 1: Agarose gel illustration of *HLA-G* 14bp Ins/Del and +3142 C/G genotyping. (a) Lane M1: 100bp ladder; lanes 1 and 2: Ins/G, Del/G; lanes 3 and 7: Negative for *HLA-G* +3142 G allele; lane 4: Del/G; lanes 5, 6, 8 and 9: Ins/G; lane N: NTC; Lane M2: 50bp ladder; N: No template control; IC: Internal control (β-globin). (b) Lane M: 100bp ladder; lane 1, 3 and 4: Del/C; lane 2: Negative for *HLA-G* +3142 C allele; lane N: No template control; IC: Internal control (β-globin)