Human Genomics

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Ralationship between polymorphisms and diplotypes of HLA-G 3'UTR and fetuses with abnormal chromosomes or unexplained pregnancy loss (UPL)

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Abstract

Objectives Human leukocyte antigen G (HLA-G) plays a crucial role in pregnancy. Pregnancy loss (PL) is caused by a variety of causes, such as fetal chromosomal abnormalities, maternal hypertension and diabetes, immune causes, spontaneous immune diseases, infections, unknown causes, etc. This study reports on the association of fetal HLA-G 3'UTR polymorphisms and diplotypes with chromosomally abnormal fetuses (CAF) or unexplained pregnancy loss (UPL).

Methods A total of 552 specimens were collected and grouped by next-generation sequencing technology (NGS) and fetal survival: UPL (112 cases), CAF (170 cases) and control (258 cases). The polymorphisms of HLA-G 3'UTR in all samples were detected by Sanger sequencing. The genotypes, haplotypes and diplotypes of HLA-G 3'UTR were analyzed. The classification and regression tree (CART) analysis was used to evaluate the role of HLA-G diplotypes in predicting fetal outcomes. The correlations between CAF or UPL and maternal age, paternal age, times of miscarrage, times of delivery were analyzed by logistic regression.

Results The frequencies of HLA-G + 2960del/del and + 3035CC genotypes were remarkablly increased in CAF than those in control group. The frequencies of HLA-G + 2960ins/del, + 3010CC, + 3035TC, + 3142GG, + 3187AA in CAF were significantly lower than those in normal fetuses. Through genetic models and logistic regression analysis, the dominant model of HLA-G 3'UTR genotypes [such as + 2960 (OR = 1.27, 95% CI = 1.05–1.54, $p = 0.016$), + 3010 (OR = 0.78, 95% CI = 0.63–0.97, $p = 0.026$), + 3035 (OR = 1.22, 95% CI = 1.00–1.49, $p = 0.047$), + 3142 (OR = 0.76, 95% CI = 0.62–0.95, $p = 0.014$) and + 3187 (OR = 0.80, 95% CI = 0.65–0.99, $p = 0.041$)] were dramatically associated with CAF. However, the frequencies of HLA-G + 3010GC, + 3142GC and + 3187AG in fetuses with UPL were memorably decreased than those in normal fetuses. No significant difference was found in the frequencies of HLA-G haplotypes in all groups. However, the frequency of UTR-1 positive specimens in CAF was significantly higher than that in UPL and control group. At the same time, the frequency of UTR-1/UTR-3 diplotypes in CAF was observably higher than that in UPL and control group, while the UTR-1/UTR-7 frequency in UPL was signally lower than that in control group. Multivariate logistic regression analysis indicated that positive HLA-G UTR-1 (OR = 1.8, 95% CI = 1.16–2.81, $p = 0.009$),

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times of abortion (OR = 1.23, 95% CI = 1.02–1.50, $p = 0.035$), and times of delivery (OR = 0.31, 95% CI = 0.20–0.48, $p < 0.001$) were correlated with CAF.

Conclusions This study suggests that HLA-G 3'UTR polymorphisms and diplotypes play an important role in the process of successful pregnancy of the embryos with abnormal chromosomes after fertilization. At the same time, Different alleles or diplotypes also affect the development of embryos with UPL.

Keywords HLA-G 3'UTR, Polymorphisms, Diplotypes, Unexplained pregnancy loss, Chromosome abnormality

Introduction

Pregnancy loss (PL) is the most serious adverse pregnancy outcome, including spontaneous abortion and stillbirth. There are many causes of PL, such as abnormal chromosomes, maternal factors (pathogenic bacterial infection, diabetes, hypertension, endocrine abnormality), immune causes, environmental factors and other factors [1, 2]. About 50% of miscarriage samples are fetal chromosomal abnormalities [3]. It is well known that maternal age is strongly correlated with chromosomal abnormalities [4]. In the meantime, The older a pregnant woman is, the less likely she is to conceive [5]. Therefore, how do the embryos with abnormal chromosomes succeed in pregnancy and how do normal fetuses lose pregnancy, which are worth studying.

A fetus is a semi-homologous graft derived half of its material from his father. A successful pregnancy requires maternal immune tolerance. If the immune tolerance is broken, it may cause pregnancy loss [6, 7]. During pregnancy, chorionic cells of the fetus and decidual cells of the mother were form a critical maternal-fetal interface. Chorionic cells are very close to decidual cells, but the immune cells in decidual cells do not attack chorionic cells because of tolerance during pregnancy [8]. However, the mechanisms that maintain or impair immune homeostasis in maternal-fetal interface remain unclear.

On account of the immune tolerance function and highly restricted in the adults, human leucocyte antigen-G (HLA-G) was widely studied in the occurrence and development of cancer, infection, autoimmune disease, recurrent spontaneous abortion (RSA) and preeclampsia (PE) [9–13]. As early as 1990, Kovats et al. found that HLA-G expressed in early human trophoblast cells, and played an important role in immune regulation during embryonic development [14]. HLA-G promoted spiral artery remodeling, immune tolerance and fetal growth during pregnancy [12].

In spite of highly restricted in the healthy adults, HLA-G was routinely expressed in the reproductive system, such as male testis, epididymis tissue, seminal plasma, mature oocytes, embryo, and so on [15–21]. The concentration of soluble HLA-G (sHLA-G) in male seminal plasma varied with the difference of HLA-G genotypes and haplotypes. High levels of sHLA-G are associated with HLA-G UTR-1 and HLA-G 14 bp deletion (del), low levels of sHLA-G are relevant to HLA-G UTR-2, UTR-4, UTR-7, and HLA-G 14 bp insertion (ins). Different sHLA-G levels may affect the quality of semen and pregnancy [16–18]. At the same time, sHLA-G cannot be detected in immature oocytes, while it can be detected in mature oocytes which helps to control the timing of in vitro fertilization [19]. However, high levels of follicular fluid sHLA-G predicted fertilization failure of the corresponding oocyte which was not associated with high-quality embryo formation [20]. In addition, sHLA-G had a positive influence on implantation and embryo survival, and might be a effective marker of embryonic development potential [21].

In terms of reproduction and immunity, the correlation between HLA-G polymorphisms (especially HLA-G 3'UTR) and RSA and PE in pregnant women were the most studied. Among them, HLA-G rs1063320 (+3142G>C) and HLA-G rs371194629 14 bp ins/del polymorphisms were the most associated with RSA and PE [22–26]. Different HLA-G genotypes or diplotypes of trophoblast cells lead to imbalance of different HLA-G mRNA and protein expression [27].

Previous studies had found that the level of sHLA-G in the sera of pregnant woman with trisomy 21 fetuses was significantly higher than that of pregnant woman with normal fetuses [28]. At the same time, the sHLA-G concentration in maternal sera with trisomy 18 fetuses was significantly lower than that of the control group [28]. The reason for lower sHLA-G in maternal sera with trisomy 18 fetuses was related to the smaller placenta. So far, no studies have been found on the relationship between HLA-G polymorphisms in CAF and fetuses with unexplained pregnancy loss (UPL).

Methods

Study design overview

A total of 552 specimens were recruited from November 2017 to March 2022 in our Taizhou Hospital of Zhejiang Province, China. There were 231 embryos of spontaneous abortion, 171 cases of amniotic fluid cells and 150 normal embryos by artificial abortion. The inclusion criteria for this study were very strict: (1) A pregnant woman or a woman who had just had a miscarriage; (2) No chronic disease (obesity, diabetes, hypertension, autoimmune diseases, etc.); (3) Normal uterus and ovaries; (4) No

abnormal immune factors such as excessive anti-phospholipid antibodies, insufficiency of blocking antibody, and so on; (5) The couple's chromosomes are normal.

All samples were tested by next-generation sequencing technology (NGS), STR genotyping and HLA-G 3'UTR analysis. Amniotic fluid cells were obtained by amniocentesis between 19 and 38 weeks of gestation. Among them, six cases of stillbirths occurred in late pregnancy, twelve cases lost to follow-up, and forty-five fetuses had abnormal chromosomes. Three groups were divided according to NGS results and fetal delivery: UPL (112 cases), CAF (170 cases) and control (258 cases). The research strategy was shown in Fig. 1 and the information for each groups was shown in Table 1. This study was approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province, and all women provided informed consent in accordance with the Declaration of Helsinki.

DNA extraction

Genomic DNA was isolated from embryonic tissue, fetal tissue and amniotic fluid cells using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the instructions.

STR genotyping

The embryonic tissue, fetal tissue and amniotic fluid cells were compared with the maternal peripheral blood by STR genotyping to rule out maternal contamination or unqualified specimens. Human DNA typing kit (Yan Huang, BGI) was used. In strict accordance with the kit

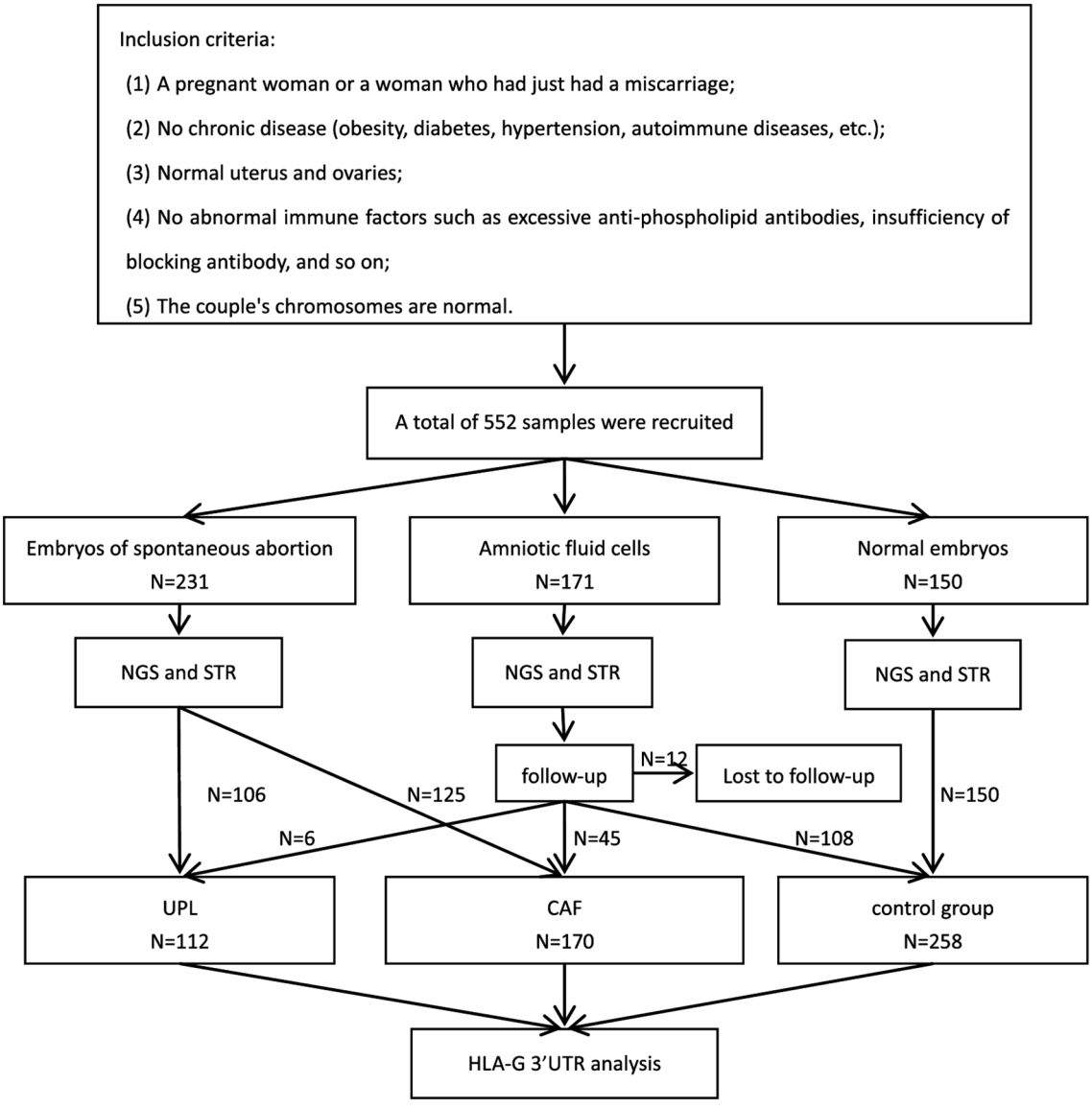

Fig. 1 Flowchart of research strategy

Table 1 Sample information

Characteristic	UPL	CAF	Control
	N = 112	*N* = 170	*N* = 258
Maternal age, years			
Median (IQR)	29.0 (26.3, 32.0)	31.5 (27.8, 35.3)	30.0 (26.0, 33.2)
Range	20–41	21–45	18–46
Paternal age, years			
Median (IQR)	31.0 (28.3, 34.0)	33.0 (29.0, 36.0)	32.0 (28.0, 34.0)
Range	22–48	19–51	20–48
Times of pregnancies			
Median (IQR)	2 (1, 3)	2 (1, 3)	2 (1, 3)
Range	1–9	1–10	1–8
Times of abortions			
Median (IQR)	1 (0, 2)	1 (0, 2)	0 (0, 1)
Range	0–7	0–9	0–5
Times of delivery			
Median (IQR)	0 (0, 1)	0 (0, 1)	1 (0, 1)
Range	0–3	0–2	0–3
Gender of the fetuses			
Boy (XY)	48 (42.9)	80 (47.1)	121 (46.9)
Girl (XX or XO)	64 (57.1)	90 (52.9)	137 (53.1)

No statistical difference was found in all data

instructions, the final test was carried out with the ABI 3730XL sequencer.

Next-generation sequencing technology (NGS)

DNA was used for library construction (Ion plus Fragment Library Adapters Kit, Life Technologies, United States) and the NGS detection was done using the Ion Proton Sequencer (400 flows, Life Technologies, United States). The effective results of NGS were that sequencing depth was greater than 15X and usable reads were more than 5 Million. Trisomy or monomer of a chromosome and deletion or repetition of large fragment (>10 Mb) were judged as abnormal chromosomes.

HLA-G 3'UTR analysis

Polymerase chain reaction (PCR) and Sanger sequencing were used for HLA-G 3'UTR analysis. A 467 bp or 453 bp of DNA fragment was amplified. Primer design, reaction system and amplification program were shown in supplementary Table 1. PCR products were purified and forward primers were used for sequencing using ABI 3730Xl sequencer (Life technologies, United States). The quality was strictly controlled by adding negative control and random double-blind repeated experiment. To ensure the accuracy of the results, all samples were reverse-sequenced to rule out false positives and false negatives.

Statistical analysis

Spss22.0 statistical software was used to calculate the distribution frequencies of alleles and genotypes. Hardy-Weinberg equilibrium of all genotypes were calculated in both experimental groups and control group. If the measurement data were in normal distribution, median ± standard deviation (SD) and t-test were used, If not, median (interquartile, IQR) and rank-sum test were used. The counting data were described by number (percentage), and the chi-square test was used. Linkage disequilibrium (LD) of single nucleotide polymorphisms (SNPs) of HLA-G 3'UTR was performed by using PowerMarker 3.25 and Haploview 4.2. D' and r^2 were used to describe the LD degree between each SNPs. According to the literature, the allele with higher frequency was referred to major allele "A" and the other was minor allele "a" [29]. The associations of genotypes in the HLA-G 3'UTR with CAF or UPL were analyzed by logistic regression using genotype genetic models, such as heterozygous (AA vs. Aa), homozygous (AA vs. aa), dominant (AA vs. Aa+aa), recessive (aa vs. AA+Aa) and additive (AA vs. Aa vs. aa) models [29].

HLA-G 3'UTR haplotypes were analyzed. Fetal HLA-G 3'UTR polymorphisms and haplotypes which impacted CAF or UPL were predicted by classification and regression tree (CART) analysis. The ratio of analysis set to test set was 8:2. The correlations between CAF or UPL and maternal age, paternal age, times of miscarrage, times of delivery were analyzed by logistic regression. A $p<0.05$ was considered to be markedly significant. Assuming an allele frequency of 0.612 (observed for HLA-G+3142GC) in CAF, group sizes of 80 in each group would be adequate to detect an allelic odds ratio of at least 0.41, at a power of 80% and significant level of 5%.

Results

No maternal contamination

The maternal contamination was assessed with STR genotyping. The results showed that there was no maternal contamination in embryonic tissue, fetal tissue and amniotic fluid cells (supplementary Fig. 1).

NGS results

A total of 231 fetuses were aborted in the first trimester, of which 125 (54.1%) had abnormal chromosomes by NGS (Fig. 1). Human has 23 pairs of chromosomes, most fetal chromosome abnormalities could occur PL. Among them, the incidence of fetal trisomy 16 caused PL was the highest (Fig. 2). There were 45 fetuses who had abnormal chromosomes by amniocentesis in the second trimester, and the incidence of trisomy 21 is the highest (Fig. 2). Therefore, the 170 fetuses with abnormal chromosomes were classified into CAF. Three hundred and seventy cases had normal chromosomes. PL with normal chromosomes in utero were classified as UPL (112 cases). 258 cases with normal chromosomes and successful delivery were used as control group.

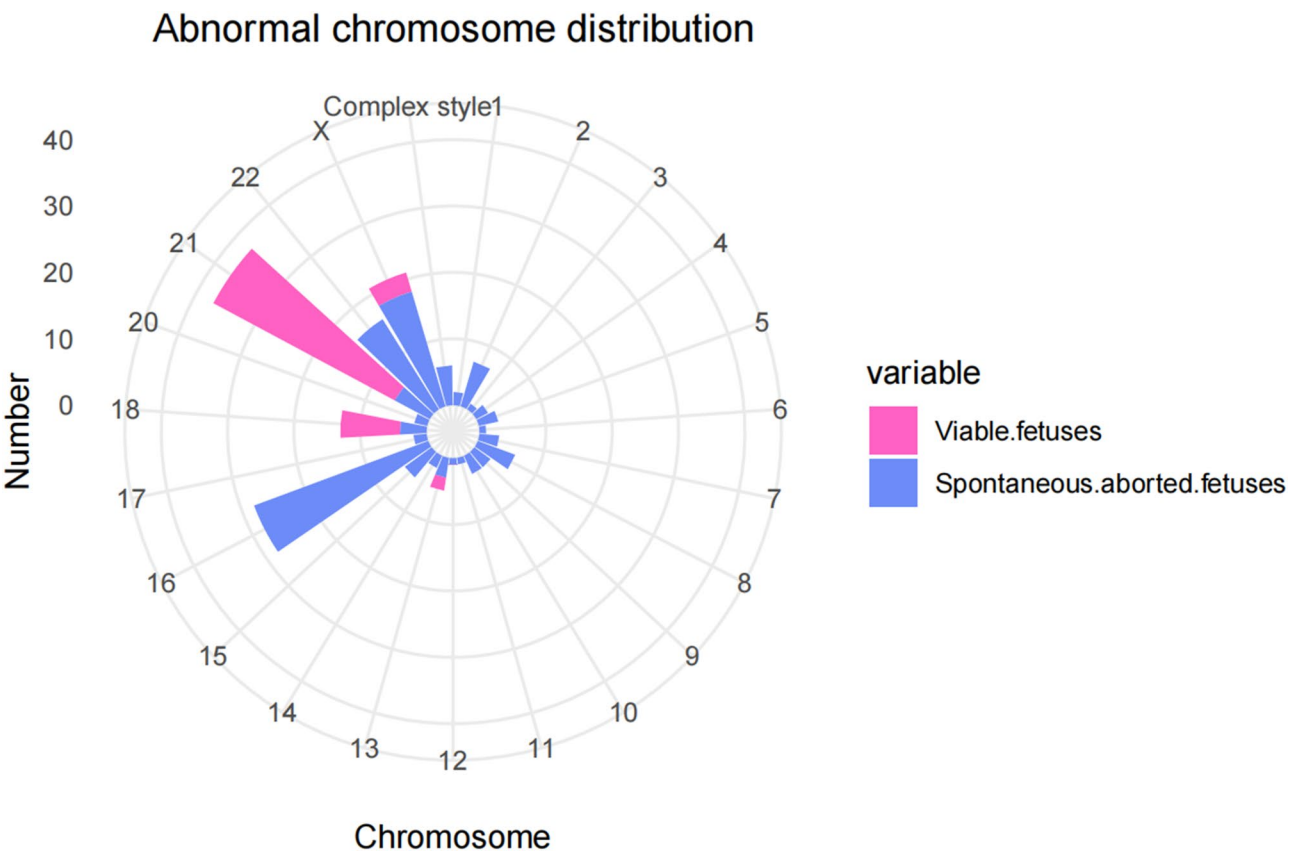

Fig. 2 The distribution of abnormal chromosomes in CAF

HLA-G 3'UTR genotypes and allelic frequencies

According to SNP website (https://www.ncbi.nlm.nih.gov/snp/), there were 19 polymorphic sites in HLA-G 3'UTR. However, only 8 SNPs of HLA-G 3'UTR existed in the southern Chinese population and eight haplotypes were formed (supplementary Fig. 2A and B).

In UPL and control group, all genotypes of HLA-G 3'UTR were in line with hardy-Weinberg equilibrium. Natural selection (high mortality) occurred in CAF, resulting in dissatisfying with Hardy-Weinberg equilibrium (such as HLA-G+3010, +3142, +3187 and +3196) (Table 2)

We found significant differences in the frequencies of HLA-G 3'UTR genotype between UPL, CAF and control group. All p value, OR and 95% confidence intervals (95% CI) were shown in Table 2. The frequencies of HLA-G 3'UTR+3010CC genotype (24.7% vs. 40.2% and 34.9%), +3142GG genotype (24.7% vs. 42.9% and 36.0%) and +3187AA genotype (27.6% vs. 42.9% and 37.2%) in CAF were significantly lower than those in UPL and control group (all $p<0.05$). However, the frequencies of +3010GC genotype (42.0% vs. 61.2% and 53.5%), +3142GC genotype (39.3% vs. 61.2% and 52.3%) and +3187AG genotype (40.2% vs. 60.0% and 51.6%) in UPL was markedly lower than those in CAF and control group (all $p<0.05$). The frequencies of +2960ins/del (37.6% vs. 49.2%) and +3035TC (32.4% vs. 41.9%) in CAF was dramatically lower than control group (all $p<0.05$). However, the frequencies of +2960del/del (54.1% vs. 42.2%) and +3035CC (64.7% vs. 55%) in CAF was observably higher than control group (all $p<0.05$).

Notably, there were no enormous differences in the HLA-G 3'UTR genotypic frequencies between the viable fetuses and spontaneous aborted fetuses with abnormal chromosomes (supplementary Table 2).

Through genetic model and logistic regression analysis, HLA-G 3'UTR genotype heterozygous model and dominant model [such as +2960 (OR=1.68 and 1.27), +3010 (OR=0.62 and 0.78), +3035 (OR=1.52 and 1.22), +3142 (OR=0.59 and 0.76) and +3187 (OR= 0.64 and 0.80), all $p<0.05$] were remarkable significance between CAF and control group (See Table 3 for details). However, there was no statistical difference between UPL and control group.

HLA-G 3'UTR haplotype and diplotypes

Linkage disequilibrium (LD) analysis showed that there were four pairs of variants showing higher allelic association: +3027A/C and +3035 C/T alleles ($r^2=0.96$, D' = 1.00), +3010 C/G and +3142 C/G alleles ($r^2=0.97$, D' = 0.99), +3010 C/G and +3187 A/G alleles ($r^2=0.92$, D' =

Table 2 The genotype and minor allele frequencies of HLA-G 3'UTR from UPL, CAF and control group

Genotype		UPL	CAF	Control	UPL vs. CAF			UPL vs. Control			CAF vs. Control		
		N = 112	*N* = 170	*N* = 258	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
+2960ins (rs371194629)													
ins/ins		11(9.8)	14(8.2)	22(8.5)	0.647	1.21	0.53–2.78	0.688	1.17	0.55–2.50	0.915	0.96	0.48–1.94
ins/del		50(44.6)	64(37.6)	127(49.2)	0.241	1.34	0.82–2.17	0.418	0.83	0.53–1.30	**0.018**	**0.62**	**0.42–0.92**
del/del		51(45.5)	92(54.1)	109(42.2)	0.158	0.71	0.44–1.14	0.558	1.14	0.73–1.79	**0.016**	**1.61**	**1.09–2.38**
Minor allele ins		72(32.1)	92(27.1)	171(33.1)	0.193	1.28	0.88–1.85	0.791	0.96	0.68–1.34	0.059	0.75	0.55–1.01
H-W	χ^2	0.061	0.364	3.168									
	p	0.97	0.834	0.205									
+3010 (rs1710)													
GG		20(17.9)	24(14.1)	30(11.6)	0.397	1.32	0.69–2.53	0.107	1.65	0.89–3.06	0.448	1.25	0.70–2.22
GC		47(42)	104(61.2)	138(53.5)	**0.002**	**0.46**	**0.28–0.75**	**0.042**	**0.63**	**0.40–0.98**	0.116	1.37	0.92–2.03
CC		45(40.2)	42(24.7)	90(34.9)	**0.006**	**2.05**	**1.22–3.42**	0.331	1.25	0.79–1.98	**0.026**	**0.61**	**0.40–0.94**
Minor allele G		87(38.8)	152(44.7)	198(38.4)	0.168	0.79	0.56–1.11	0.904	1.02	0.74–1.41	0.065	1.30	0.98–1.71
H-W	χ^2	1.525	9.581	4.423									
	p	0.466	**0.008**	0.11									
+3027 (rs17179101)													
AA		5(4.5)	4(2.4)	7(2.7)	0.324	1.94	0.51–7.38	0.382	1.68	0.52–5.40	0.818	0.86	0.25–3.00
AC		41(36.6)	55(32.4)	106(41.1)	0.461	1.21	0.73–1.99	0.419	0.83	0.52–1.31	0.068	0.69	0.46–1.03
CC		66(58.9)	111(65.3)	145(56.2)	0.279	0.76	0.47–1.25	0.626	1.12	0.71–1.75	0.060	1.47	0.98–2.19
Minor allele A		51(22.8)	63(18.5)	120(23.3)	0.220	1.30	0.86–1.96	0.885	0.97	0.67–1.41	0.099	0.75	0.53–1.06
H-W	χ^2	0.187	0.871	5.883									
	p	0.911	0.647	0.053									
+3035 (rs17179108)													
TT		6(5.4)	5(2.9)	8(3.1)	0.305	1.87	0.56–6.27	0.296	1.77	0.60–5.22	0.925	0.95	0.31–2.95
TC		40(35.7)	55(32.4)	108(41.9)	0.559	1.16	0.70–1.92	0.268	0.77	0.49–1.22	**0.047**	**0.66**	**0.44–1.00**
CC		66(58.9)	110(64.7)	142(55)	0.327	0.78	0.48–1.28	0.488	1.17	0.75–1.84	**0.047**	**1.50**	**1.01–2.23**
Minor allele T		52(23.2)	65(19.1)	124(24)	0.240	1.28	0.85–1.93	0.811	0.96	0.66–1.38	0.090	0.75	0.53–1.05
H-W	χ^2	0.0003	0.362	5.536									
	p	0.9998	0.834	0.063									
+3142 (rs1063320)													
CC		20(17.9)	24(14.1)	30(11.6)	0.397	1.32	0.69–2.53	0.107	1.65	0.89–3.06	0.448	1.25	0.70–2.22
GC		44(39.3)	104(61.2)	135(52.3)	**< 0.001**	**0.41**	**0.25–0.67**	**0.021**	**0.59**	**0.38–0.93**	0.071	1.44	0.97–2.13
GG		48(42.9)	42(24.7)	93(36)	**0.001**	**2.29**	**1.37–3.81**	0.215	1.33	0.85–2.09	**0.013**	**0.58**	**0.38–0.90**
Minor allele C		84(37.5)	152(44.7)	195(37.8)	0.090	0.74	0.53–1.05	0.940	0.99	0.72–1.37	**0.044**	**1.33**	**1.01–1.76**
H-W	χ^2	2.936	9.581	3.287									
	p	0.23	**0.008**	0.193									
+3187 (rs9380142)													
GG		19(17)	21(12.4)	29(11.2)	0.248	1.45	0.74–2.84	0.132	1.61	0.86–3.02	0.726	1.11	0.61–2.02
AG		45(40.2)	102(60)	133(51.6)	**0.001**	**0.45**	**0.28–0.73**	**0.044**	**0.63**	**0.40–0.99**	0.086	1.41	0.95–2.09

Table 2 (continued)

Genotype		UPL	CAF	Control	UPL vs. CAF			UPL vs. Control			CAF vs. Control		
		N = 112	*N* = 170	*N* = 258	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
AA		48(42.9)	47(27.6)	96(37.2)	**0.008**	**1.96**	**1.19–3.25**	0.306	1.27	0.81–1.99	**0.040**	**0.65**	**0.42–0.98**
Minor allele G		83(37.1)	144(42.4)	191(37)	0.209	0.80	0.57–1.13	0.992	1.00	0.72–1.39	0.117	1.25	0.95–1.65
H-W	χ^2	2.154	8.895	2.875									
	p	0.341	**0.012**	0.237									
+3196 (rs1610696)													
GG		1(0.9)	4(2.4)	2(0.8)	0.363	0.37	0.04–3.39	0.908	1.15	0.10–12.85	0.174	3.08	0.56–17.03
GC		19(17)	19(11.2)	43(16.7)	0.164	1.62	0.81–3.23	0.944	1.02	0.57–1.85	0.114	0.63	0.35–1.12
CC		92(82.1)	147(86.5)	213(82.6)	0.323	0.72	0.37–1.38	0.923	0.97	0.54–1.74	0.279	1.35	0.78–2.33
Minor allele G		21(9.4)	27(7.9)	47(9.1)	0.550	1.20	0.66–2.18	0.908	1.03	0.60–1.77	0.552	0.86	0.53–1.41
H-W	χ2	0.0003	9.436	0.011									
	p	0.9998	**0.009**	0.994									

Value in bold indicated significant differences. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; H-W, Hardy-Weinberg equilibrium; OR, odds ratio; 95% CI, 95% confidence interval

0.99), and +3142 C/G and +3187 A/G alleles (r^2 = 0.90, D' = 0.97) (Fig. 3).

We further analyzed the haplotypes of HLA-G 3'UTR, and the results showed that the frequencies of UTR-1 and UTR-7 were slightly higher or lower in CAF than that in UPL and control group, but there were not statistically significant (Table 4). Furthermore, we found that the presence rate of UTR-1 haplotype in CAF (72.4%) was significantly higher than that in UPL (54.5%, p = 0.002, OR = 2.19, 95% CI = 1.33–3.61) and control group (61.6%, p = 0.022, OR = 1.63, 95% CI = 1.07–2.48). (Table 5).

At the same time, HLA-G 3'UTR diplotypes were analyzed in this study. The most common diplotypes in UPL, CAF and control group were UTR-1/UTR-1 (17.0%, 12.4% and 11.2%), UTR-1/UTR-3 (19.6%, 33.5% and 22.9%), UTR-1/UTR-7 (11.6%, 18.2% and 20.2%), and UTR-3/UTR-7 (15.2%, 8.8% and 13.6%). The UTR-1/UTR-3 diplotype frequency in CAF were markedly higher than that in UPL and control group (p < 0.05, Table 6). Besides, the frequency of the UTR-1/UTR-7 diplotype in UPL group were observably lower than that in the control group (p = 0.047, Table 6).

Classification and regression tree (CART) analysis

The results of CART showed that HLA-G diplotypes, maternal age and the number of delivery played an important role in predicting CAF and UPL (Fig. 4). Using CART to analyze the test set, the prediction accuracy for CAF was 68.63% and for UPL was 71.56%.

Logistic regression analysis

Multivariate logistic regression analysis showed that maternal age, times of abortion, times of delivery and positive HLA-G UTR-1 were dramatically correlated with CAF (Table 7). Maternal age was closely related to fetal chromosomal abnormalities, and the older the age, the higher the risk factor. In order to study the relationship between HLA-G polymorphisms and CAF, the maternal age factor was excluded. Therefore, there was no significant difference in maternal age between CAF and control. Notably, positive UTR-1 was also significantly associated with CAF (OR = 1.80, 95% CI = 1.16–2.81, p = 0.009). The times of miscarriages is a disadvantage for CAF and UPL fetuses (OR = 1.23, 95% CI = 1.02–1.50, p = 0.035; OR = 1.35, 95% CI = 1.09–1.67, p = 0.006), while the times of pregnancy is a protective factor for them (OR = 0.31, 95% CI = 0.20–0.48, p < 0.001; OR = 0.22, 95% CI = 0.13–0.38, p < 0.001).

Discussion

Main findings

To the best of our knowledge, this is the first time that the HLA-G 3'UTR has been detected in embryos or

Table 3 The associations of genotype genetic models in the HLA-G 3'UTR with UPL and CAF were analyzed by logistic regression

Genotype	UPL	CAF	Control	Model	UPL vs. Control			CAF vs. Control		
	N = 112	*N* = 170	*N* = 258		*p*	OR	95% CI	*p*	OR	95% CI
+2960ins (rs371194629)				Heterozygous	0.468	1.19	0.75–1.90	**0.013**	**1.68**	**1.11–2.52**
ins/ins	11 (9.8)	14 (8.2)	22 (8.5)	Homozygous	0.870	0.97	0.65–1.44	0.445	1.15	0.80–1.66
ins/del	50 (44.6)	64 (37.6)	127 (49.2)	Dominant	0.558	1.07	0.856–1.34	**0.016**	**1.27**	**1.05–1.54**
del/del	51 (45.5)	92 (54.1)	109 (42.2)	Recessive	0.688	1.17	0.55–2.50	0.915	0.96	0.48–1.94
				Additive	0.782	1.05	0.74–1.49	0.054	1.36	1.00–1.85
+3010 (rs1710)				Heterozygous	0.123	1.47	0.90–2.39	**0.035**	**0.62**	**0.40–0.97**
GG	20 (17.9)	24 (14.1)	30 (11.6)	Homozygous	0.400	0.87	0.62–1.21	0.124	0.76	0.55–1.06
GC	47 (42)	104 (61.2)	138 (53.5)	Dominant	0.331	1.12	0.89–1.41	**0.026**	**0.78**	**0.63–0.97**
CC	45 (40.2)	42 (24.7)	90 (34.9)	Recessive	0.110	1.65	0.89–3.06	0.448	1.25	0.70–2.22
				Additive	0.902	0.98	0.70–1.36	**0.044**	**0.73**	**0.54–0.99**
+3027 (rs17179101)				Heterozygous	0.491	1.18	0.74–1.87	0.062	1.48	0.98–2.22
AA	5 (4.5)	4 (2.4)	7 (2.7)	Homozygous	0.456	0.80	0.44–1.44	0.647	1.16	0.62–2.17
AC	41 (36.6)	55 (32.4)	106 (41.1)	Dominant	0.626	1.06	0.85–1.32	0.061	1.21	0.99–1.48
CC	66 (58.9)	111 (65.3)	145 (56.2)	Recessive	0.387	1.68	0.52–5.40	0.818	0.86	0.25–3.00
				Additive	0.878	1.03	0.69–1.54	0.080	1.39	0.96–1.99
+3035 (rs17179108)				Heterozygous	0.339	1.26	0.79–2.00	**0.044**	**1.52**	**1.01–2.29**
TT	6 (5.4)	5 (2.9)	8 (3.1)	Homozygous	0.393	0.79	0.46–1.36	0.713	1.11	0.63–1.97
TC	40 (35.7)	55 (32.4)	108 (41.9)	Dominant	0.489	1.08	0.87–1.36	**0.047**	**1.22**	**1.00–1.49**
CC	66 (58.9)	110 (64.7)	142 (55)	Recessive	0.302	1.77	0.60–5.22	0.925	0.95	0.31–2.95
				Additive	0.800	1.05	0.71–1.55	0.074	1.39	0.97–1.98
+3142 (rs1063320)				Heterozygous	0.064	1.58	0.97–2.57	**0.019**	**0.59**	**0.38–0.92**
CC	20 (17.9)	24 (14.1)	30 (11.6)	Homozygous	0.450	0.88	0.63–1.23	0.084	0.75	0.54–1.04
GC	44 (39.3)	104 (61.2)	135 (52.3)	Dominant	0.216	1.15	0.92–1.45	**0.014**	**0.76**	**0.62–0.95**
GG	48 (42.9)	42 (24.7)	93 (36)	Recessive	0.110	1.65	0.89–3.06	0.448	1.25	0.70–2.22
				Additive	0.939	1.01	0.73–1.41	**0.029**	**0.71**	**0.52–0.97**
+3187 (rs9380142)				Heterozygous	0.114	1.48	0.91–2.40	**0.043**	**0.64**	**0.41–0.99**
GG	19 (17)	21 (12.4)	29 (11.2)	Homozygous	0.432	0.87	0.62–1.22	0.246	0.82	0.59–1.14
AG	45 (40.2)	102 (60)	133 (51.6)	Dominant	0.306	1.13	0.90–1.41	**0.041**	**0.80**	**0.65–0.99**
AA	48 (42.9)	47 (27.6)	96 (37.2)	Recessive	0.135	0.31	0.86–3.02	0.726	1.11	0.61–2.02
				Additive	0.992	1.00	0.72–1.39	0.090	0.77	0.57–1.04
+3196 (rs1610696)				Heterozygous	0.940	0.98	0.54–1.77	0.131	1.56	0.88–2.79
GG	1 (0.9)	4 (2.4)	2 (0.8)	Homozygous	0.905	0.93	0.28–3.11	0.223	0.59	0.25–1.38
GC	19 (17)	19 (11.2)	43 (16.7)	Dominant	0.923	0.99	0.74–1.32	0.280	1.16	0.89–1.53
CC	92 (82.1)	147 (86.5)	213 (82.6)	Recessive	0.908	1.15	0.10–12.85	0.196	3.08	0.56–17.03
				Additive	0.908	0.97	0.56–1.66	0.568	1.15	0.71–1.85

Value in bold indicated significant differences. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; OR, odds ratio; 95% CI, 95% confidence interval

amniotic fluid with CA or UPL. A total of 552 specimens were recruited for this project. They were divided into three groups: CAF (*N*=170), UPL (*N*=112) and normal controls (*N*=258), according to pregnancy outcome, NGS and STR.

The frequencies of HLA-G+3010GC, +3142GC, and +3187AG in UPL were dramatically lower than those in the CAF and control group, while the frequencies of +3010CC, +3142GG and +3187AA in CAF were memorably lower than those of the other two groups. Through genetic model and logistic regression analysis, the heterozygous model and dominant model of HLA-G 3'UTR genotype [such as +2960 (OR=1.68 and 1.27), +3010 (OR=0.62 and 0.78), +3035 (OR=1.52 and 1.22), +3142 (OR=0.59 and 0.76) and +3187 (OR=0.64 and 0.80)] were remarkable association with the CAF. There were no significant difference in the frequency of HLA-G 3'UTR polymorphisms between spontaneously aborted embryos and viable fetuses in CAF and in the HLA-G 3'UTR haplotype among the three groups.

The positive rate of UTR-1 in CAF group was observably higher than that in the other two groups. At the same time, the UTR-1/ UTR-3 diplotypes frequency in CAF group was significantly higher than that in UPL and control group, while the UTR-1/UTR-7 frequency in UPL group was significantly lower than that in control group. Through CART analysis, it was found that HLA-G 3'UTR diplotype play an important role in predicting

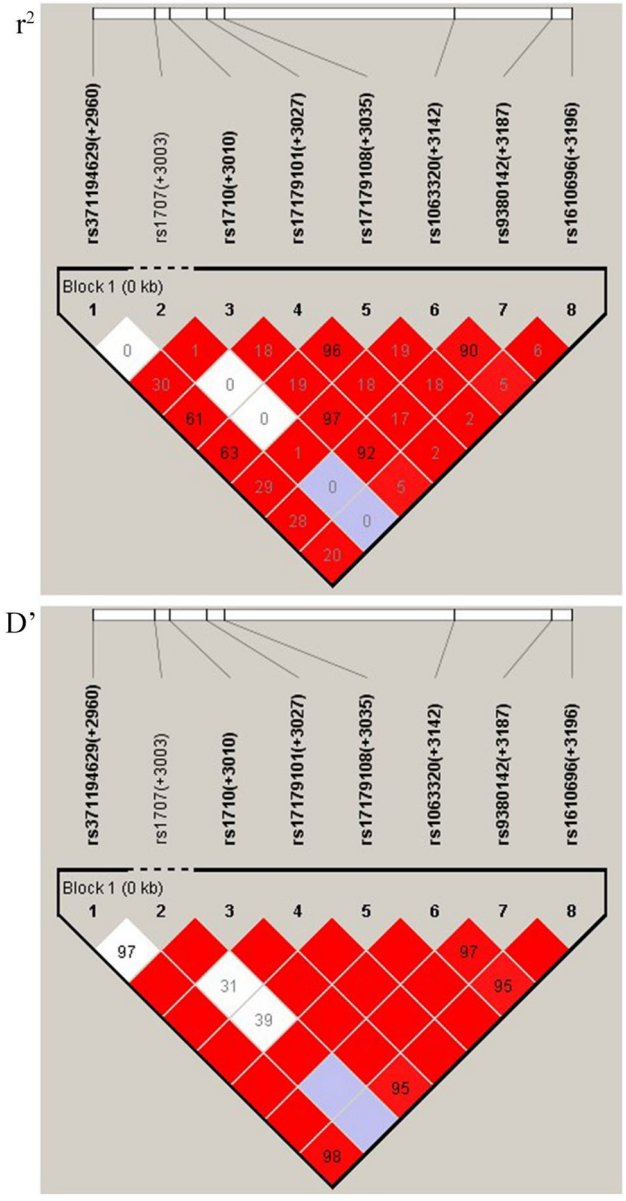

Fig. 3 The results of HLA-G 3'UTR gene Linkage disequilibrium analysis (Haploview 4.2). D'and r^2, pairwise correlation between polymorphic sites

UPL and CAF, respectively. Multivariate logistic regression analysis showed that positive HLA-G UTR-1, times of abortion, and times of delivery were dramatically correlated with CAF.

At present, there are a lot of studies on genetic polymorphisms of pregnant women with recurrent spontaneous abortion (RSA), but few studies on genetic polymorphisms of the fetuses itself after spontaneous PL. A survey was conducted to record the stress and depression of pregnant women after the first PL, and to analyze the relationship between stress or depression and recurrent PL (RPL) in the following two years. The results suggested that stress and depression might be risk factors for

Table 4 HLA-G 3'UTR haplotype frequencies in UPL, CAF and control group

Haplotypes	UPL	CAF	control	UPL vs. CAF			UPL vs. control			CAF vs. control		
	N = 224	*N* = 340	*N* = 516	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
UTR-1 (DelTGCCCGC)	80 (35.7)	144 (42.4)	188 (36.4)	0.115	0.76	0.53–1.07	0.852	0.97	0.70–1.34	0.082	1.28	0.97–1.70
UTR-2 (InsTCCCGAG)	20 (8.9)	27 (7.9)	47 (9.1)	0.678	1.14	0.62–2.08	0.938	0.98	0.57–1.70	0.552	0.86	0.53–1.41
UTR-3 (DelTCCCGAC)	65 (29)	96 (28.2)	147 (28.5)	0.840	1.04	0.72–1.51	0.884	1.03	0.73–1.45	0.936	0.99	0.73–1.34
UTR-4 (DelCGCCCAC)	2 (0.9)	6 (1.8)	5 (1)	0.488	0.50	0.10–2.51	1.000	0.92	0.18–4.78	0.360	1.84	0.56–6.06
UTR-5 (InsTCCTGAC)	1 (0.4)	2 (0.6)	4 (0.8)	1.000	0.76	0.07–8.41	1.000	0.57	0.06–5.16	1.000	0.76	0.14–4.16
UTR-6 (DelTGCCCAC)	2 (0.9)	2 (0.6)	2 (0.4)	0.651	1.52	0.21–10.89	0.589	2.32	0.32–16.54	0.652	1.52	0.21–10.85
UTR-7 (InsTCATGAC)	51 (22.8)	63 (18.5)	120 (23.3)	0.220	1.30	0.86–1.96	0.885	0.97	0.67–1.41	0.099	0.75	0.53–1.06
other (DelTGCCGGC)	3 (1.3)	0 (0)	3 (0.6)	0.062	0.99	0.97–1.00	0.374	2.32	0.47–11.59	0.281	1.01	1.00–1.01

No significant difference was found in this data. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; OR, odds ratio; 95% CI, 95% confidence interval

Table 5 Haplotype positive distribution frequencies of HLA-G 3'UTR in UPL, CAF and control group

Diploid type	UPL	CAF	control	UPL vs. CAF			UPL vs. control			CAF vs. control		
	N = 112	*N* = 170	*N* = 258	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
UTR-1 pos	61 (54.5)	123 (72.4)	159 (61.6)	**0.002**	**0.46**	**0.28–0.75**	0.197	0.75	0.48–1.17	**0.022**	**1.63**	**1.07–2.48**
UTR-2 pos	20 (17.9)	23 (13.5)	45 (17.4)	0.323	1.39	0.72–2.67	0.923	1.03	0.58–1.84	0.279	0.74	0.43–1.28
UTR-3 pos	56 (50)	87 (51.2)	128 (49.6)	0.847	0.95	0.59–1.54	0.945	1.02	0.65–1.58	0.751	1.07	0.72–1.57
UTR-4 pos	2 (1.8)	6 (3.5)	5 (1.9)	0.484	0.50	0.10–2.51	1.000	0.92	0.18–4.82	0.357	1.85	0.56–6.17
UTR-5 pos	1 (0.9)	2 (1.2)	4 (1.6)	1.000	0.76	0.07–8.45	1.000	0.57	0.06–5.18	1.000	0.76	0.14–4.17
UTR-6 pos	2 (1.8)	2 (1.2)	2 (0.8)	0.651	1.53	0.21–11.00	0.588	2.33	0.32–16.73	0.651	1.52	0.21–10.92
UTR-7 pos	46 (41.1)	59 (34.7)	113 (43.8)	0.279	1.31	0.80–2.14	0.626	0.89	0.57–1.40	0.060	0.68	0.46–1.02
other pos	3 (2.7)	0 (0)	3 (1.2)	0.062	0.97	0.94–1.00	0.372	2.34	0.47–11.77	0.280	1.01	1.00–1.03

Value in bold indicated significant differences. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; OR, odds ratio; 95% CI, 95% confidence interval

Table 6 HLA-G 3'UTR diplotypes frequencies in UPL, CAF and control groups

Diplotypes	UPL	CAF	control	UPL vs. CAF			UPL vs. control			CAF vs. control		
	N = 112	*N* = 170	*N* = 258	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
UTR-1/UTR-1	19 (17)	21 (12.4)	29 (11.2)	0.277	1.45	0.740–2.840	0.132	1.61	0.862–3.019	0.726	1.11	0.612–2.024
UTR-1/UTR-2	6 (5.4)	10 (5.9)	17 (6.6)	0.852	0.91	0.320–2.566	0.652	0.80	0.308–2.092	0.769	0.89	0.396–1.984
UTR-1/UTR-3	22 (19.6)	57 (33.5)	59 (22.9)	**0.011**	**0.49**	**0.276–0.852**	0.491	0.82	0.476–1.428	**0.015**	**1.70**	**1.106–2.618**
UTR-1/UTR-7	13 (11.6)	31 (18.2)	52 (20.2)	0.179	0.59	0.293–1.182	**0.047**	**0.52**	**0.271–1.000**	0.623	0.88	0.539–1.448
UTR-2/UTR-3	8 (7.1)	4 (2.4)	13 (5)	0.070	3.19	0.938–10.867	0.422	1.45	0.583–3.602	0.164	0.45	0.146–1.417
UTR-3/UTR-3	9 (8)	9 (5.3)	19 (7.4)	0.357	1.56	0.601–4.068	0.822	1.10	0.481–2.511	0.397	0.70	0.310–1.593
UTR-3/UTR-7	17 (15.2)	15 (8.8)	35 (13.6)	0.100	1.85	0.882–3.875	0.682	1.14	0.609–2.135	0.135	0.62	0.326–1.168
other haplogenotype	18 (16.1)	23 (13.5)	34 (13.2)	0.553	1.22	0.627–2.389	0.462	1.26	0.679–2.345	0.917	1.03	0.584–1.820

Value in bold indicated significant differences. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; OR, odds ratio; 95% CI, 95% confidence interval

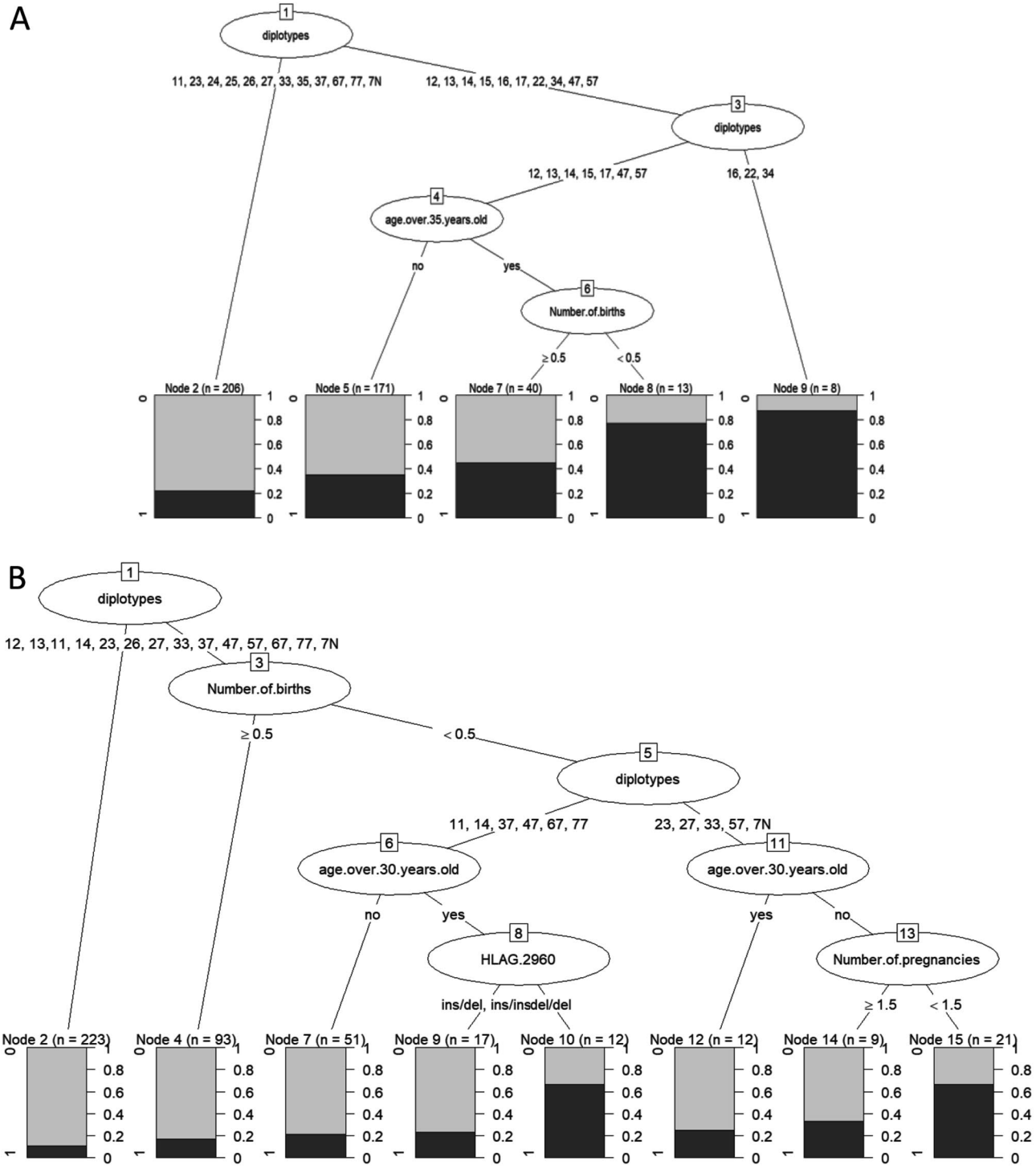

Fig. 4 The results of classification and regression tree (CART) analysis. (**A**) The results of CART showed that HLA-G diplotypes, maternal age and the number of birth played an important role in predicting CAF. (**B**) The results of CART showed that HLA-G diplotypes, the times of delivery, maternal age, and HLA-G + 2960 and the times of pregnancy played an important role in predicting UPL. The black area represented the experimental group, and the gray area represented the control group. The ratio of analysis set to test set was 8:2. Using CART to analyze the test set, the prediction accuracy for CAF was 68.63% and for UPL was 71.56%

Table 7 Multivariate logistic regression for the association of UPL or CAF with various factors

Characteristics	UPL vs. control			CAF vs. control		
	p	OR	95% CI	*p*	OR	95% CI
maternal age	0.708	1.02	0.93–1.11	0.053	1.07	1.00-1.15
Paternal age	0.641	1.02	0.94–1.11	0.438	1.03	0.96–1.09
times of abortion	**0.006**	**1.35**	**1.09–1.67**	**0.035**	**1.23**	**1.02–1.50**
times of delivery	**< 0.001**	**0.22**	**0.13–0.38**	**< 0.001**	**0.31**	**0.20–0.48**
Gender of the fetuses	0.426	1.21	0.75–1.95	0.058	1.42	0.99–2.03
UTR-1 pos	0.257	0.76	0.47–1.22	**0.009**	**1.80**	**1.16–2.81**

Value in bold indicated significant differences. UPL, the fetuses with unexplained pregnancy loss; CAF, chromosomally abnormal fetuses; OR, odds ratio; 95% CI, 95% confidence interval

RPL in pregnant women [30]. Studying the causes of PL from the perspective of the fetuses may be more effective in alleviating anxiety and depression in pregnant women.

Zhu et al. [31] showed that 56.9% (1355/2383) of fetuses had pathogenic CA among the RPL. In the embryos of spontaneous abortion of this study, the proportion of pathogenic CA fetuses was 54.1% (125/231), which was very close to the results of the above study. Trisomy 16 had the highest incidence in early spontaneous abortion embryos. Studies had found that the expression of HLA-G in spontaneous aborted fetuses induced by trisomy 16 or with normal chromosomes was significantly decreased from that in embryos by selective abortion [32]. However, this article did not consider the gestational age of the embryos, and simply compared the HLA-G expression level which might have errors.

HLA-G 3'UTR polymorphisms (especially+3142 C/G and +2960ins/del), haplotypes and diplotypes might affect the level of sHLA-G, and might be predictors of cancer susceptibility and prognosis [33–35]. In women with cervical cancer, the frequency of HLA-G 3'UTR haplotype was not different from that of normal women, but UTR-1/UTR-3 diplotype frequency was significantly higher than that of normal control group [33]. In patients with breast cancer, UTR-7 haplotype is associated with low tumor burden, and UTR-4 is associated with tumor size (>T1) and poor prognosis [34]. At present, few studies have studied HLA-G polymorphisms in embryos or fetuses in PL. In this study, it was found that UTR-1 may play an important role in the survival of ovums or sperms with abnormal chromosomes, and the successful implantation of blastocysts (especially UTR-1/UTR-3) after fertilization. Different abnormal chromosomes would lead to surviving or spontaneously aborting of embryos. The role of HLA-G in this might be to keep the fetuses alive as much as possible. Meantime, The low frequency of UTR-1/UTR-7 diplotype might be harmful to UPL.

As everyone known, age and the times of miscarriage were disadvantages to pregnancy. Cellular aging, as women aged, leaded to age-related declines in reproductive function [36]. There was a large number of studies in China showing that the increase in the times of abortion could lead to secondary infertility. In addition, fetal chromosomal abnormalities could lead to an imbalance in the expression of certain genes, which could lead to its fatal death [37]. sHLA-G had played an important role when the embryo implanted in the uterus [13–15]. HLA-G UTR-1 haplotype, 14 bp deletion allele, and +3142 G allele were significantly associated with high concentration of sHLA-G [38–40]. This was consistent with the research in this paper. HLA-G protected the CAF from surviving for some time in the face of numerous adverse factors.

This study has some limitations. First of all, due to the low frequency of +3003TC, a large number of samples are needed to analysis. Therefore, this SNP site was deleted during the analysis in this paper. Second, on account of the lack of timely ultrasound examination, fetal intrauterine abortion may be observed later than the actual occurrence. Some pregnant women may choose to prevent miscarriages for 1 to 2 weeks. Surgery was not done until the embryo had atrophied or remains without a fetal heart. Therefore, the concentration of sHLA-G in pregnant women's plasma and the gestational weeks were incorrect. It resulted in elimination of the sHLA-G results. Third, in consideration of the inaccuracy of gestational age, it was impossible to study the correlation on HLA-G diplotype and fetal survival time in utero.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40246-024-00695-5.

Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Danping Xu was responsible for study design, article writing and statistic analysis. Yiyang Zhu was in charge of NGS detection. Jun Wang took charge of the polymorphism detection of HLA-G 3'UTR. Heqin Guan were responsible for explaining and signing informed consent with patients and collecting specimens. Xiuzhen Shen was responsible for DNA extraction and STR detection.

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Data availability
The experimental data has been uploaded to dbVar database (SUB13964903).

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Ethics Review Committee of Taizhou Hospital (approval #K20231035), and written informed consent from all study participants was obtained before enrolment.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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