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*Original Article*

# Clinical relevance of sHLA-G to premature rupture of membrane

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**Abstract:** This study aims to explore potential clinical relevance of sHLA-G to premature rupture of membrane. 75 samples of pregnancy were enrolled, including patients with preterm premature rupture of membranes (PPROM), patients with term premature rupture of membranes (TPROM) and normal pregnancy women. Level of sHLA-G in multiple sites were examined with ELISA. Lymphocytes were isolated from peripheral blood of patients with premature rupture of membrane and cultured *in vitro*. Lymphocytes proliferation was detected with MTT assay. Interestingly, no significant difference in the level of sHLA-G in serum and amniotic fluid was detected between PPROM group and TPROM group (P>0.05). In PPROM group and TPROM group, the level of sHLA-G in HCS lesion was higher than non-HCA lesion. Increasing incidence of HCA was associated with longer duration of membrane rupture. Regression analysis indicated duration of membrane rupture was an independent influencing factor for composites of premature rupture of fetal membranes and HCA, suggesting that pregnancy women with PROM should adopt early parturition to prevent HCA prolonged by duration of membrane rupture. MTT assay showed high concentration of HAMSC inhibited lymphocytes proliferation, while MEM-G/9Ab attenuated HAMSC inhibiting effect on lymphocytes proliferation. In summary, premature rupture of fetal membranes should be considered if decreasing level of HAMSC was detected. Inhibiting lymphocytes proliferation had promising clinical relevance in PROM.

**Keywords:** sHLA-G, premature rupture of fetal membranes, histologic chorioamnionitis, mesenchymal cells

## Introduction

Premature rupture of fetal membranes (PROM) is an important obstetrical complication, but its cause and mechanism are still not well-known, including preterm premature rupture of membranes (PPROM) and term premature rupture of membranes (TPROM). Incidence of PPROM was 10% while TPROM was 3.5% [1-3]. 30%-40% of PROM patients werte complicated by premature delivery, placental abruption, fetal distress, prolapse of cord and neonatal respiratory distress syndrome. Moreover, incidences of such complications are growing each year, which are important causes leading increased perinatal infection and perinatal death. As a PROM complication of concern, chorioamnionitis is characterized by infection of amniotic fluid, fetal membranes and placenta and jeopardizes pregnancy health [4, 5]. Early diagnosis for pregnancy women with PROM will help to determine optimal therapy for termination of pregnancy so as to reduce incidences of perinatal infection and perinatal death. PROM has various pathogenesis, and most are interaction of multiple factors. Despite of many studies conducted in recent years, causes of PROM still remains unclear, and precise marker for PROM complicated by chorioamnionitis is warranted. As a novel immune tolerance factors, sHLA-G belongs to nonclassical HLA-1 antigen [6]. Researches indicated normal expression of sHLA-G in trophoblasts was important for maintaining healthy pregnancy. Our study was focused on the level of HLA-G in serum and amniotic fluid in the population of pregnancy women with PROM. Lymphocytes from TPROM and PPROM were cultured in vitro to explore effect of sHLA-G on PROM and related immunization in HAMSC. We aimed to provide data for clinical treatment and prevention.

## Materials and methods

### *Materials*

*Materials source:* 50 PROM (TPROM and PPROM) pregnancy and 25 naturally delivered preg-

nancy with intact membrane treated in obstetrics from August 2013 to August 2014 were studied. 25 cases in TPROM were 26.4 ± 2.8 years old, gestational weeks: 37~41, average 39 weeks, and another 25 cases in PPROM were 27.5 ± 2.9 years old, gestational weeks: 30~36, average 32 weeks. The membrane of those two group were detected in pathology department of our hospital, the membrane were divided into HCA (histologic chorioamnionitis) and non-HCA (non-chorioamnionitis). The normal delivery whose membranes were well were treated as control (no chorioamnionitis after detection), average 25 ± 2.8 years old, 37~41 gestational weeks and average 39 weeks. The age and weight index of PPROM, TPROM and normal group distributed equilibrium, there were no difference between those group and comparable.

*Inclusion criteria:* (1) primipara were detected as single live births by colorful Doppler ultrasound; (2) exclusion infection result from other reasons; (3) without gestational hypertension and diabetes; (4) exclusion other medical and surgical diseases; (5) signature the informed consent.

*Diagnostic criteria:*(1) PROM diagnose (1) patients consciously found the urine liquid flowing out the vaginas; (2) colposcopy: when liquid flowing from cervix, effusion was found in back vault, or fetal effusion mixed in the visible liquid; (3) vaginal fluid pH determination: normal pH4.5~5.5, amniotic fluid pH 7.0~7.5, vaginal fluid detection: pH>6.5 suggest the broken membrane; (4) vaginal smears detection: the effusion extracted by puncture from vagina dome and detected under the microscopy, smear showed sheep dentate crystallization after drying; (5) color Doppler examination, the amniotic fluid volume reduced. (2) The pathological diagnostic criteria of the histologic chorionic amniotic membrane inflammation the placenta chorionic plate showed dark color under microscope and neutrophil infiltration.

*Method*

Primary reagents and instruments HLA-G enzyme-linked immunosorbent assay kit (Shanghai jing combines biological technology co). 550 type enzyme-linked immunometric meter (Bio-Rab). HLA-G antibody (MEM-G/9) was used (Bio-Rab); MTS (Bio-Rab).

Sample collection membrane broken time of patients was recorded and serum, amniotic fluid and membrane tissues were collected of premature rupture of fetal membrane patients.

*Venous blood collection:* 5 ml venous blood (without any anticoagulant)were collected from PPROM, TPROM and normal labor, who hospitalized without any treatment, the blood solidified naturally at room temperature for 20 min, supernatant was collected after centrifuging for 20 min (3000 r/min), then saved in -20°C.

*Amniotic fluid collection:* normal vagina sterilizing detection were performed, making the amniotic fluid flowing from the vagina by thrusting the fetal head, then 5 ml amniotic fluid were collected by disposable syringe. However, 5 ml amniotic fluid were collected from normal delivery after amniotic membrane exposure when they delivery. The collected amniotic fluid was loaded into bacterium-free tubes, collecting the supernatant following the same method above, preserved at -20°C.

*Fetal membrane collection:* 2×2 cm fetal membrane tissue full-thickness were collected after the three group patient delivery, 3, 6, 9, 12 piont distant from fetal membrane creva-sse 5 cm were collected, washing by sterile saline solution, randomly selecting a block of membrane, then fixed by formaldehyde and inspected.

*HAMSC isolation and generation culture of cells [7]*

The normal delivery membrane of the cord surface were blunt dissected in bacteria-free environment, and dissected by operating scissors, 10×10 cm membrane was uniformly taken out and treated as experiment specimen. The blood and cell debris were washed away by PBS and digested by 2.5 g/L trypsin for twice, 10 minutes every time. The rest tissues were digested in 1.0 g/L collagenase for 120 min at 37°C, and filtered into single-cell suspension, samples were collected after centrifuging, living cells were counted by trypan blue. Then DMEM/F12 (containing 10% FBS and 20 μg/L bFGF), then $2\times10^5$/ml cells were seeded into 75 ml culture flask and cultured in 37°C, 5% $CO_2$ incubator, culture solution was changed after 48 h, next the culture was changed every 3 days. When cell fusion was above 30%, digestion was

performed by adding 2.5 g/L trypsin and cultured by passaging.

*ELISA detection of HLA-G [8]*

HAMC supernatant of 3-5 generations cell cultured for 3 d was centrifuged for at 3000 r/min, then supernatant was slowly sucked up by 0.5 L micro pipette tip. Strictly based on the steps of enzyme-linked assay kit, sHLA-G ELISA kit was used for serum and amniotic fluid sHLA-G detection.

*MTT assay of lymphocyte proliferation [9]*

30 ml peripheral blood was collected from randomly selected 3 normal deliveries, and lymphocyte were collected by Ficoll solution, washing twice and resuspended by RPMI 1640 (10% FBS, after culturing 12 h at 37°C, the adherent cells in culture fiask were removed for further operation, then the cells were fully treated by 50 ug/ml third mitomycin C about 1 h. cells were seeded on the basis of ectomesenchymal cell villus density in 96 wells plates, culturing 24 h in constant temperature environment, supernatant was slowly removed by micro pipette tip, then adding stimulated lymphocytes into the wells by using the same pipette tip (density 2×104).

Cells were divided into 7 groups, 2×104 concentration amniotic mesenchymal stem cells were added into the first group, based on the first group, 100 mg/ml HLA-G antibody was added into the second group; 2×103 concentration amniotic mesenchymal stem cells were added into the third group based on the first group; based on the third group, the same HLA-G antibody as the second group was added into the fourth group; the fifth group was only added the amniotic mesenchymal stem cells supernatant based on the first group, the equal quantity HLA-G antibody as the second and forth group was added into the sixth group, and the seventh group treated as control with other reagents and inductor.

20 μLMTS was added into the seven groups cells cultured for 3 d, then incubating for 4 h and $A_{450m}$ was detected, inhibition ratio was calculated repeatedly for 3 times. Inhibition ratio=[control absorbance value ($A_{450m}$)-treatment absorbance value ($A_{450m}$)/control absorbance ($A_{450m}$)] ×100%.

*Statistical analysis*

All results were analyzed with SPSS19.0, measurement data are normal distribution to X ± S, F test was used for comparative analysis of different groups, and t test was used for comparison of two groups. All the relevant measurement data was tested by chi-square. *P* value <0.05 was considered to be statistically significant.

## Results

*Examination of sHLA-G in PPROM group and TPROM group*

Compared with normal pregnancy group (control group), sHLA-G of PPROM group was significantly higher, both in serum (T=4.434, P<0.05) and amniotic fluid (T=4.299, P<0.05). Compared with normal pregnancy group, sHLA-G of TPROM group was significantly higher, both in serum (T=5.528, P<0.05) and amniotic fluid (T=2.422, P<0.05). Moreover, no statistical difference was detected in the level of serum sHLA-G between TPROM group and PPROM group (T=0.288, P>0.05) same as the level of amniotic fluid sHLA-G (T=3.150, P>0.05) (**Table 1**) .

*Levels of sHLA-G in HCA lesion and non-HCA lesion*

Levels of serum sHLA-G and amniotic fluid sHLA-G in HCA lesion, whether PPROM group or TPROM group, were significantly higher than those in non-HCA lesion (P<0.05) (**Table 2**) .

*Association between duration of membrane rupture and incidence of chorioamnionitis*

We analyzed numbers of fetal membrane infection and duration of membrane rupture. 29 samples had <8 h duration of membrane rupture, 17 samples 8-12 h, 15 samples >12 h. Incidences of chorioamnionitis were showed in **Table 3**, of which patients with >12 h duration of membrane rupture had highest incidences, and statistical differences were detected ($\chi^2$=24.137, P<0.05). Regression analysis indicated duration of membrane rupture was an independent influencing factor for composites of premature rupture of fetal membranes and HCA (OR=6.528, 95% CI 4.422 ± 0.039, T=3.92, P<0.05).

**Table 1.** Analysis of serum sHLA-G and amniotic fluid sHLA-G among three group

| Group | Samples | serum sHLA-G (ng/ml) | amniotic fluid sHLA-G (ng/ml) |
|---|---|---|---|
| PPROM | 25 | 39.12 ± 3.19① | 58.39 ± 3.68① |
| TPROM | 25 | 38.67 ± 4.55② | 58.22 ± 2.49② |
| Control | 25 | 33.19 ± 3.30 | 50.61 ± 5.28 |

①and ②represent comparison with control group (P<0.05).

**Table 2.** Analysis of sHLA-G in HCA lesion and non-HCA lesion (X ± S)

| Group | Samples | serum sHLA-G (ng/ml) | amniotic fluid sHLA-G (ng/ml) |
|---|---|---|---|
| TPROM | 25 | | |
| HCA | 8 | 42.38 ± 1.25① | 60.79 ± 2.15① |
| Non-HCA | 16 | 36.01 ± 1.39 | 54.08 ± 0.21 |
| PPROM | 25 | | |
| HCA | 12 | 41.65 ± 1.58① | 61.68 ± 1.33① |
| Non-HCA | 13 | 36.19 ± 1.55 | 54.54 ± 0.56 |

①represents comparison between HCA and non-HCA (P<0.05).

**Table 3.** Analysis of association between duration of membrane rupture and incidence of chorioamnionitis

| Group | Sample | Positive | Negative | Incidence (%) | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| <8 h | 29 | 3 | 26 | 10.3 | 24.137 | 0.001 |
| 8~12 h | 16 | 5 | 11 | 31.25 | 15.240 | 0.028 |
| >12 h | 15 | 10 | 5 | 66.7 | 5.399 | 0.033 |

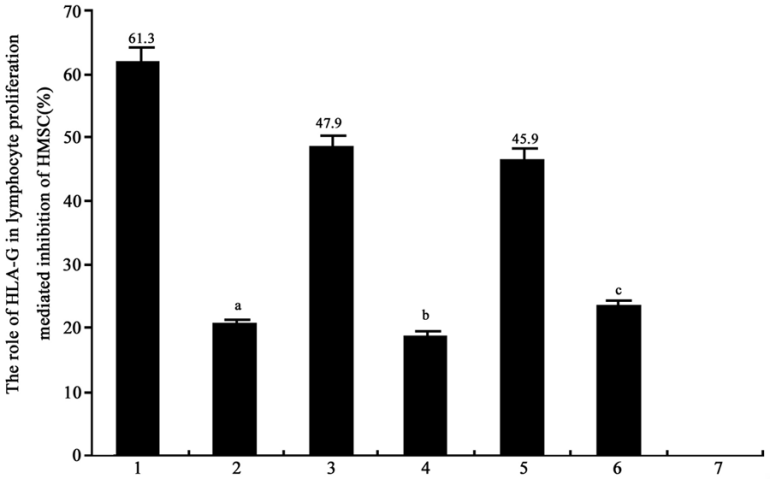


**Figure 1.** HAMSC-induced inhibiting effect on lymphocytes proliferation in each group (X ± SD, n=3).

*Inhibiting effect on lymphocytes proliferation*

Inhibition rates were examined in 7 groups (**Figure 1**). Results showed both HAMSC and MEM-G/9Ab were influencing factors for inhibition rates. HAMSC inhibited lymphocytes proliferation and such inhibiting effect was concentration-dependent, while MEM-G/9Ab attenuated HAMSC inhibiting effect on lymphocytes proliferation (**Table 4**).

## Discussion

PROM is one of common obstetrics complications. Lots of studies proved PROM the result of the mutual-function of many factors from both macro and micro aspects. Infection was the most common pathological factor, such as multiple vaginal inflammatory ascending infection or intraamniotic infection. Rati-os of chorioamnionitis and obstetric infection were 13%~60% and 2%~13% in clinical scenario, respectivly. 33.3% of patients with HCA were diagnosed by pathoogical examination, while 66.7-% were misdiagnosed or never diagnosed due to asymtomatic symptoms or suclinical chorioamnionitis, consequently, early diagnosis is difficult [10-12].

As a member of major histocompatibility complex, HLA-G belongs to nonclassical HLA-1 antigen. Geraghty et al. firstly cloned human HLA-G with relative molecular weight (37~39)×$10^3$. Its transcription products could be transformed with multiple splicing forms into four different membrane-bound human leukocyte antigens (m-HLA-G1, G2, G3, G4) and three different soluble membrane molecules (sHLA-G5, G6, G7), which were characterized with low polymorphism and limited distribution. HLA-G was mainly distributed in extravillous cytotrophoblasts of maternal-fetal interface, and maintained pregnancy.

**Table 4.** Analysis of inhibiting effect on lymphocytes proliferation

| independent variable | correlation | SD | T | P | 95% CI |
|---|---|---|---|---|---|
| HAMSC concentration① | 8.602 | 3.260 | 5.184 | <0.05 | 1.330~0.252 |
| HAMSC② | 6.528 | 0.147 | 2.021 | <0.05 | 0.266~0.102 |
| MEM-G/9Ab③ | -11.528 | 4.392 | -2.144 | >0.05 | -1.295~-0.035 |

①represents analysis among group 1, group 3 and group 5. ②represents analysis among 7 groups. ③represents analysis among group 1 to group 6.

Some studies indicated success rate of pregnancy was associated with the level of sHLA-G in pregnant women. Higher level of sHLA-G suggested promising pregnancy. sHLA-G was involved in maintaining maternal-fetal tolerance and functions of many kinds of immune cells. sHLA-G regulated immunity of maternal-fetal interface, therefore, ab-normal expression of sHLA-G suggested pathologic pregnancy, such as preeclampsia and recurrent spontaneous abortion. Researchers found levels of sHLA-G in different period of pregnancy were significant lower than non-pregnancy period, including sHLA-G in amniotic fluid, serum and umbilical cord blood, of which sHLA-G in amniotic fluid was of the most significance. Accordingly, sHLA-G influenced more widely than membrane HLA-G [13-15]. Our study showed sHLA-G, whether in amniotic fluid or serum, of PPROM group and TPROM group were higher than control group (P<0.05). Moreover, sHLA-G in amniotic fluid was higher than serum. All above suggested sHLA-G was highly expressed in pregnancy women with PROM and resulted in maternal-fetal immune tolerance, which was consistent with previous study [16].

sHLA-G was probably involved in HCA host immune response regulation. The elevation of sHLA-G concentration repressed immune cell viability and finally caused HCA Compared with HCA lesion in PPROM group and TPROM group, sHLA-G of amniotic fluid and serum was lower in non-HCA lesion, more importantly, such change preceded symptoms of chorioamnionitis and subclinical chorioamnionitis, suggesting sHLA-G examination was of great clinical importance to diagnosis of PROM complicated with HCA. Moreover, level of HCA was not affected by other factors, for instance, gestational age. Therefore, sHL-A-G could be included in HCA early event as one of clinical diagnostic factors for chorioamnionitis [17]. Inc-reasing incidence of HCA was associated with longer duration of membrane rupture, and HAC likely caused premature delivery and fetal intrauterine infection. Our study showed patients with HCA lesion had higher level of sHLA-G of amniotic fluid and serum. Further investigation explored association between duration of membrane rupture and incidence of HCA, and showed incidence of HCA in patients with <8 h duration of membrane rupture was 10.3%, 31.25% in 8-12 h, 66.7% in >12 h. Statistical difference was confirmed with FISHER test (P<0.01). Our results indicated duration of membrane rupture was an independent influencing factor for composites of premature rupture of fetal membranes and HCA, suggesting pregnancy women with PROM should adopt early parturition to prevent HCA prolonged by duration of membrane rupture, which was consistent with previous study [18]. Accordingly, in pregnancy women without significant clinical infection symptoms, sHLA-G of amniotic fluid and serum should be monitored for differential diagnosis of suspected HCA. In other hand, sHLA-G was quite conducive to PROM patient and use of antibiotics for premature delivery to prevent medical malpractice and reduce mortality of perinatal infants.

As a member of multipotential stem cells, amniotic mesenchymal stem cells induce amniotic immunoreaction with low immunogenicity and high immunosuppressive function [19]. Current experiment in vitro showed different inhibition rates in seven groups. HAMSC and MEM-G/9Ab were influencing factors for inhibition rates. A positive correlation was proved between the concentration of HAMSC and lymphocyte proliferation inhibition rate, suggesting HAMSC significantly influenced lymphocyte proliferation inhibition rate, which was consistent with what Kinoshita T et al. found [20]. Consequently, we confirmed high concentration of amniotic mesenchymal stem cells in pregnancy women with PROM, and HAMSC should be monitored in clinical examination to prevent PROM. Moreover, intergroup analysis among group 1 to group 6 showed MEM-G/9Ab decreased lymphocyte proliferation inhibition rate, suggesting lymphocyte proliferation inhibition rate was a potential target for PROM preventive treatment.

Our study also showed inhibiting effect of HAMSC on lymphocyte proliferation was associated with cell count, and inhibition rate dropped after HLA-G specific antibody intervention. Potential mechanism was lymphocyte proliferation could be reduced by soluble cytokines secreted from amniotic mesenchymal stem cells, while HLA-G specific antibody repressed such inhibiting effect. Meanwhile, our study indicated HLA-G not only inhibited HAMSC induced immunoreaction, but inhibited lymphocyte proliferation to promote PROM and HCA, accordingly, sHLA-G should be of concern for PROM preventive treatment.

In conclusion, our study confirmed inhibiting effect of HLA-G on HAMSC cellular immunity. Pregnancy women with PROM had higher sHLA-G levels, both in serum and amniotic fluid. To pregnancy women, sHLA-G was an important clinical inspection item for early diagnosis of HCA lesion or PROM to reduce maternal infection rate and perinatal mortality. PROM should be considered if decreasing level of HAMSC was detected. Inhibiting lymphocytes proliferation had promising clinical significance in the field of PROM drug therapy.

## Disclosure of conflict of interest

None.

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