Original Article

Novel nucleic acid aptamer gold (Au)-nanoparticles (AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2) to detect the soluble human leukocyte antigen G5 subtype (HLA-G5) in liquid samples

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Background: The human leukocyte antigen G5 subtype (HLA-G5) is a major histocompatibility complex (MHC) molecule that is selectively expressed at the maternal-foetal tissue interface and is required for the successful implantation of the *in vitro* fertilized embryo. It is critical to detect HLA-G5, especially HLA-G5 expression in embryo fluid, during *in vitro* embryo incubation and culture. However, the specificity and sensitivity of traditional ELISA methods to detect sHLA-G5 are insufficient. This work aimed to explore novel nucleic acid aptamer gold (Au)-nanoparticles to detect soluble HLA-G5 in liquid samples.

Methods: Soluble HLA-G5 was obtained using a prokaryotic expression system, and two novel aptamers (HLA-G5-Apt1 and HLA-G5-Apt2) detecting HLA-G5 were screened by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method. Small (10 nm) gold nanoparticles (AuNPs) were incubated with AptHLAs to form two novel nucleic acid aptamers: Au-nanoparticles (AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2).

Results: The results showed that AptHLA-G5-1 and AptHLA-G5-2 have a high affinity for HLA-G5 and can detect its presence in liquid samples. Using the colorimetric sensing method, AuNPs-AptHLA-G1 had a detection limit as low as 20 ng/mL (recovery range between 98.7% to 102.0%), while AuNPs-AptHLA-G2 had a detection limit as low as 20 ng/mL (recovery range between 98.9% to 103.6%).

Conclusions: Our work demonstrates that novel AuNPs are efficient detectors for HLA-G5 and are useful for diagnosis and treatment in the field of obstetrics-gynaecology.

Keywords: Human leukocyte antigen G5 subtype (HLA-G5); aptamer; Systematic Evolution of Ligands by Exponential Enrichment (SELEX); small gold nanoparticles; obstetric gynaecology

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Introduction

Human leukocyte antigen G5 subtype (HLA-G5) is an essential human histocompatibility complex [major histocompatibility complex (MHC)], which belongs to the Ib subtype of MHC (1-3). As an important regulatory factor of the immune system, HLA-G5 can alleviate the damage caused by verification, alleviate the rejection caused by the suppression of homologous and allologous immune-rejection, and play a protective role in the body (4-6). It is essential that HLA-G5 is selectively expressed in maternal-foetal interface tissues, where it is mainly distributed in the extravillous cytotrophoblast, and can play a protective role during embryonic development and pregnancy (7-9). In *in vitro* fertilization (IVF) embryo culture medium, human embryos can secrete soluble HLA-G protein (soluble HLA-G5, sHLA-G5) at the 4–8-cell stage, and this secretion is closely

related to the successful implantation of *in vitro* fertilized embryos (10,11). Therefore, it is critical to detect HLA-G5, especially HLA-G5 expression in embryo fluid, during *in vitro* embryo incubation and culture. However, in commonly used embryo culture medium (approximately 12 μL), the specificity and sensitivity of traditional ELISA methods to detect sHLA-G5 are insufficient. Additionally, specific limitations, such as cumbersome operation and multiple steps, hamper its use for efficient detection. Therefore, exploring the ligands that can recognize sHLA-G5 with high specificity and high affinity is of great importance to improve the specificity and sensitivity of its detection.

Nucleic acid aptamers (aptamers) are single-stranded oligonucleotides that can specifically bind to proteins or other biological macromolecules obtained from a random single-stranded oligonucleotide library (12-14). Aptamers are designed with colloidal gold nanotechnology, making them a portable, fast, simple, and highly-sensitive detection method (15,16). Gold nanoparticles were incubated with aptamers screened in this study to obtain aptamer nano-gold detection reagents (AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2). When HLA-G5 is present in a liquid test sample, it competitively binds to the aptamer in the detection reagent, causing the aptamer to dissociate from the gold nanoparticles. The use of novel AuNPs to detect HLA-G5 will assist both IVF research and the broader field of diagnostic and therapeutic procedures in obstetrics and gynaecology. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-3334).

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of the First Medical Center of Chinese PLA General Hospital (approval No.: s2019-196-01). The informed consent was waived due to the anonymous data and non-intervention feature of the study.

Materials and reagents

All the reagents used in the procedure of prokaryotic expression and purification of HLA-G5 protein were produced by the BBI Life Sciences Corporation (Shanghai, China) including the LB Broth Agar Medium, Rosetta competent bacteria, protein expression inducer, non-pre-stained protein marker, pre-stained protein marker, TMB chromogenic Kit, Western Blot primary antibody, Western Blot secondary antibody, and the protein concentration quantitative Kit. Reagents used for the ligand system evolution and aptamer screening included Dynabeads MyOne™ Carboxylic Acid (Thermo, USA), DPBS (BBI, USA), BSA (BBI, USA), NHS (Sigma, USA), EDC (J&K Scientific, China), Pfu DNA polymerase (BBI, USA), 2X high fidelity PCR mix premix (BBI, USA), sodium acetate (BBI Life Sciences Corporation, China), ethanolamine (BBI Life Sciences Corporation, China), TEMED (BBI Life Sciences Corporation, China), and dialysis bag 3.5KD (BBI Life Sciences Corporation, China).

Prokaryotic expression system of HLA-G

The pET28a vector containing the full-length region of HLA-G5 was cloned into a recombinant plasmid and transduced to Rosetta (DE3) *Escherichia coli* (*E. coli*), which was then cultured. The culture medium was continuously tested during the shaking process, and when the O.D. value at 600 nm reached 0.6, the inducer IPTG (Isopropyl-beta-D-thiogalactopyranoside) (100 mmol/L) was added, and the culture was continued at 20 ℃ for 16 h. The bacteria were collected by centrifugation then lysed, and the samples were prepared for SDS-PAGE. The separation and purification of HLA-G5 was performed using Ni-NTA (Ni column affinity chromatography packing) affinity chromatography methods (17,18), and the purified HLA-G5 protein was analyzed using SDS-PAGE.

SELEX methods to prepare the aptamer of HLA-G5

The aptamer of HLA-G5 was screened from a DNA library using methods from the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (19-22). Briefly, the carboxyl magnetic beads were incubated with HLA-G5 protein (concentration of 0.35 mg/mL) for 40 min on a shaker at room temperature. The DNA library was then diluted and distributed into PCR tubes for denaturation, and mixed with carboxyl magnetic beads combined with HLA-G5. Lastly, the carboxyl magnetic beads were separated from the system by centrifugation. The potential aptamer (the ssDNA) with HLA-G5 from the DNA library was identified using qPCR.

Detection of HLA-G based on AuNPs-aptamer sensing method

Gold nanoparticles (AuNPs) were synthesized by citrate

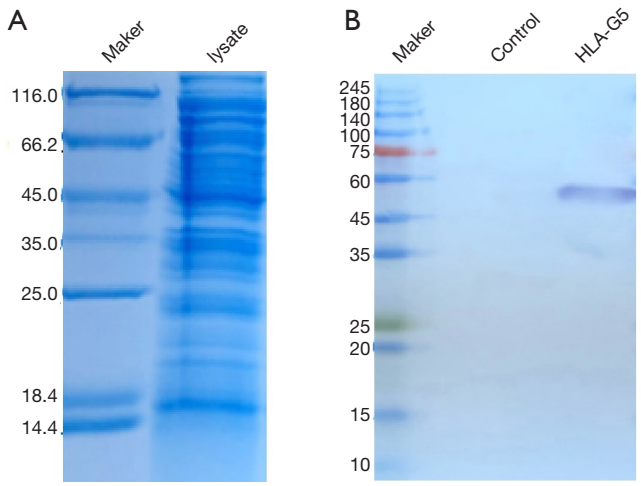

Figure 1 Purification of HLA-G5 from *E. coli*. The vectors containing the full length of HLA-G5 were transduced into *E. coli*. HLA-G5 was purified from the bacteria. (A) The lysate of the bacteria or (B) the purified HLA-G5 was analyzed by the SDS-PAGE. HLA-G5, human leukocyte antigen G5 subtype.

reduction following the methods described by Zhang [2021] and Xia [2020] (23,24). A 2% chloroauric acid solution (200 mL) was boiled under stirring conditions, and 6 mL of 2% trisodium citrate solution was quickly added. The solution was kept heated for 15 min under stirring conditions until the colour became wine red, and the prepared AuNPs solution was then stored at 4 ℃ and protected from light. Transmission electron microscope (TEM) was used to examine the prepared gold nanoparticles (25,26). A concentration gradient for HLA-G was prepared using a standard solution (10, 20, 50, 100, 200, 500, and 1,000 nmol/L) and stored at 4 ℃ for later use. An incubation buffer (Na_2HPO_4-KH_2PO_4, pH 7.6, 50 mmol/L Na^+, 5 mmol/L K^+, 5 mmol/L Mg^{2+}, 1 mmol/L Ca^{2+}) was used to dilute HLA-G5-Apt1 and HLA-G5-Apt2, and 50 μL AuNPs solution was mixed with a 4 μmol/L aptamer solution (25 μL) at room temperature for 5 min. About 25 μL of HLA-G5 standard solution with a concentration gradient was then incubated at room temperature for 20 min, and at the end of the reaction, 10 μL of NaCl solution (900 mmol/L) was added and mixed well. Colour changes were observed by absorbance changes at 520 and 650 nm were measured using a microplate reader.

Statistical analysis

The continuous variables are expressed as mean ± standard deviation (SD). The results come from three replicates with similar trends were analyzed by using the linear regression analysis. All the statistical analyses were performed by using the IBM® SPSS® Statistics software Version 22.

Results

HLA-G proteins obtained from the prokaryotic expression system

HLA-G5 proteins were obtained using the prokaryotic expression system, and successfully induced HLA-G expression in *E. coli* (*Figure 1*). The results of Coomassie brilliant blue staining showed that the HLA-G band was visible, and the molecular weight of the band was correct. Images of bacterial lysate and the purification of HLA-G protein are shown in *Figure 1A*,*1B*, respectively.

Aptamers of HLA-G5 with high affinity

The target protein (HLA-G5) was successfully coupled to the protein detection chip, and a surface plasmon resonance (SPR) test was performed. Affinity binding of HLA-G5 protein to the aptamer [HLA-G5-1 (>Seq2_778_289773_0.0027, TGTTGACCAGGGACTGCTCGGGATTGCGGATGTCAA) or HLA-G5-2 (>Seq5_368_289773_0.0013, TGTTGACCAGGGACTGCTCGGGATTGCGGACTTCAA)] is shown in the SPR curves (*Figure 2*), and the single curve shown above fits the KD value (affinity). The value corresponding to KD below *Figure 2* is the affinity dissociation constant. For HLA-G5-Apt1 (AptHLA-G5-1 $=6.356\times10^{-7}$ μmol/L), and for HLA-G5-Apt2 (AptHLA-G5-2 $=2.949\times10^{-7}$ μmol/L). This demonstrates that HLA-G5 aptamers with high affinity were successfully obtained.

Use of Au-nano-particles to examine HLA-G5

To verify the accuracy and reliability of the HLA-G detection method using nano-gold-aptamer colorimetric sensing, the aptamer and nano-Au were mixed to form the novel aptamer Au-nanoparticles AuNPs-AptHLA-G1 and AuNPs-AptHLA-G2 (*Figure 3*), and transmission electron microscopy show each had a uniform particle size of about 10 nm (*Figure 4*). HLA-G5 was detected by AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2 using a colorimetric sensing method and in liquid samples (*Figure 5*). The AuNPs-AptHLA-G5-1 colorimetric sensing method had a detection limit of approximately

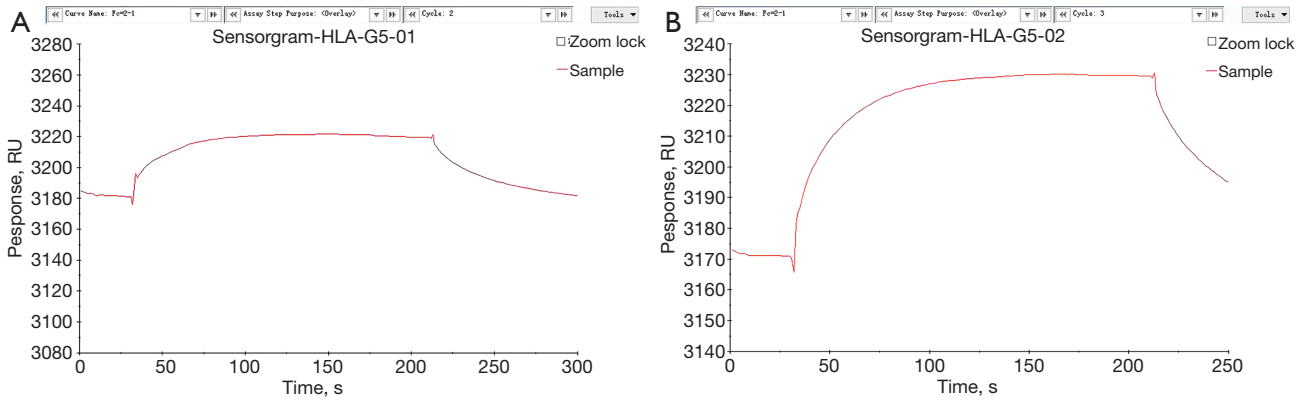

Figure 2 SPR methods were used to examine the affinity between AptHLA-G5-1 or AptHLA-G5-2 with HLA-G5. The results are shown as affinity-curves between AptHLA-G5-1 (A) with HLA-G5 or AptHLA-G5-2 with HLA-G5 (B). SPR, surface plasmon resonance; HLA-G5, human leukocyte antigen G5 subtype.

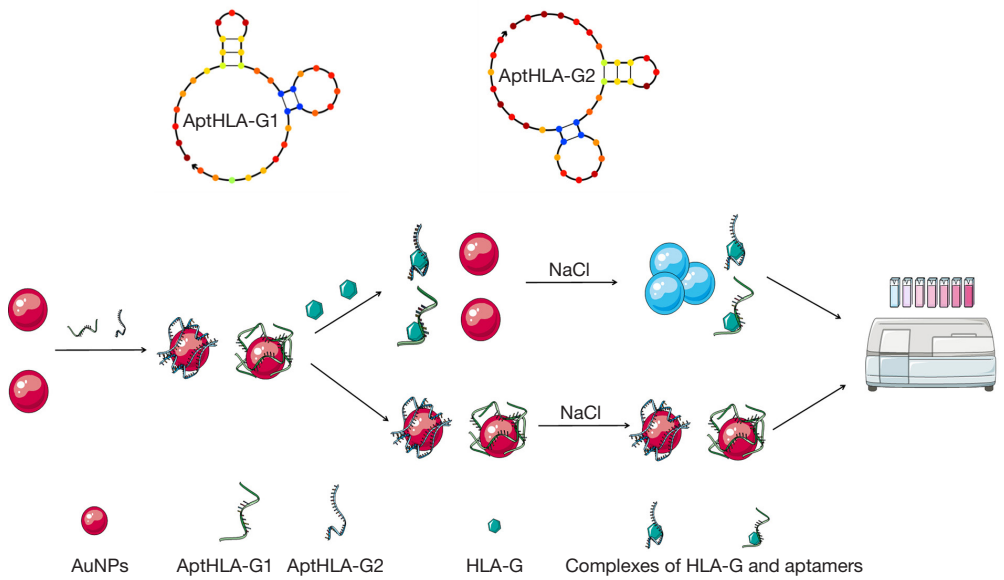

Figure 3 Principle diagram of AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2 colorimetric sensing method for detecting HLA-G. The secondary structure of aptamer is predicted by NUPACK (http://www.nupack.org/).

20 ng/mL (recovery range: 98.7% to 102.0%) while that of AuNPs-AptHLA-G5-2 was as low as 20 ng/mL (recovery range: 98.9% to 103.6%) (*Figure 5*). The standard-curves of AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2 obtained by incubation with HLA-G5 are shown in *Figure 5* and *Table 1*.

Discussion

With the influence of many factors such as social structure, population aging, environmental pollution, late marriage, and late childbirth, countries worldwide have generally seen a decline in marriage and fertility rates and a gradual increase in the incidence of fertility disorders (26,27). In addition, the gradual implementation of the Chinese governments comprehensive liberalization of the second-child policy after 2016 has seen some families who may have lost their only child or had only one child with genetic diseases or defects, to attempt an additional pregnancy using strategies such as assisted reproduction.

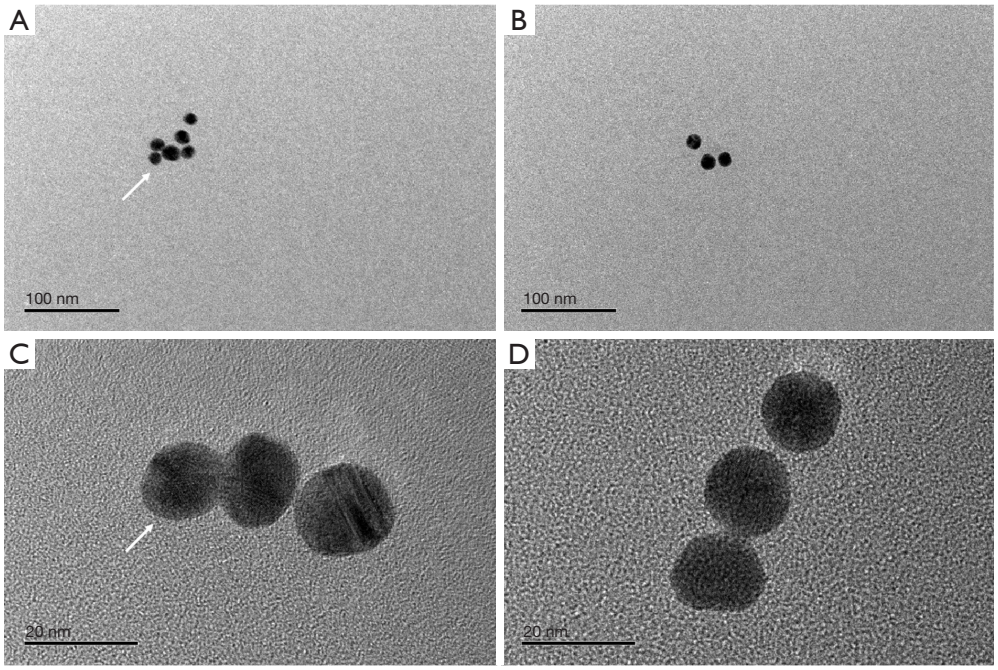

Figure 4 Transmission electron microscopy images of AuNPs-AptHLA-G1 and AuNPs-AptHLA-G2. AuNPs-AptHLA-G1 (A,C) and AuNPs-AptHLA-G2 (B,D) were examined by transmission electron microscopy. Images are shown as 100 nm scale (A,B) and 20 nm scale (C,D). The arrows indicate the nanoparticles.

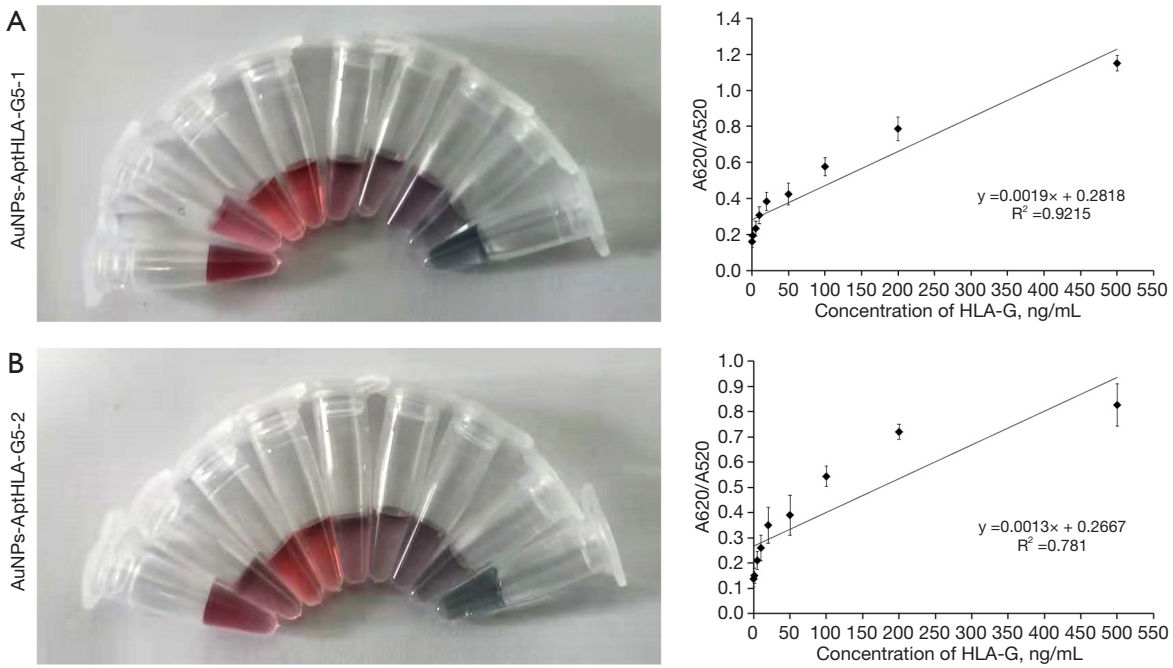

Figure 5 Novel nucleic acid aptamer Au-nanoparticles, AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2, used to detect soluble HLA-G5 in liquid samples. (A) Images of AuNPs-AptHLA-G5-1 detect the HLA-G5 in liquid samples, the standard curves of the interaction between AuNPs-AptHLA-G5-1, or the indicated concentration of HLA-G5. (B) Images of AuNPs-AptHLA-G5-2 detect the HLA-G5 in liquid samples, the standard curves of the interaction between AuNPs-AptHLA-G5-2, or the indicated concentration of HLA-G5. HLA-G5, human leukocyte antigen G5 subtype.

Table 1 Examination of AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2 of HLAG-5 in the liquid samples

Au-nano particles	HLA-G5 (ng/mL)	Testing results (ng/mL)	Recovery rate (%)	Recovery rate SD (%, n=3)
AuNPs-AptHLA-G5-1	20	20.24±1.21	101.2	5.9
	50	49.35±3.16	98.7	6.4
	100	99.80±9.02	99.8	9
	200	204.00±15.32	102	7.4
AuNPs-AptHLA-G5-2	20	20.72±1.13	103.6	5.4
	50	50.70±3.22	101.4	6.3
	100	98.90±6.15	98.9	6.2
	200	198.00±10.28	99	5.2

HLA-G5, human leukocyte antigen G5 subtype.

The most effective assisted reproduction technology at present remains in vitro fertilization (IVF), which is mainly used in cases of (I) fallopian tube disease (tubal blockage caused by pelvic inflammatory disease); (II) endometriosis and other endometrial diseases; (III) male oligospermia, asthenospermia, or teratospermia; and (IV) conditions where there is a high risk of genetic diseases or genetic defects (28,29). Despite advancements in IVF-related technology for diagnosis and treatment (to the third generation), its success rate is affected by the success rate of embryo transfer and the survival rate of foetus delivery, and remains low (approximately 20%) (30,31). For this reason, researchers are eager to develop new strategies to improve the IVF success rate and determine indicators of success. Studies have shown that single-cell omics research before embryo transfer can help solve this problem (28,32,33), but this technique requires the extraction of a small number of cells and tissues from the embryo, which may damage it (28,32,33). Since multiple pregnancies are unfavourable to the health of mothers and infants, choosing a single embryo for transplantation without reducing the pregnancy rate can effectively prevent their occurrence (34,35). However, the challenge plaguing researchers and first-line clinicians has always been selecting embryos that can better implant and survive throughout the pregnancy. At present, a widely used strategy in clinical diagnosis and treatment is to select embryos for transfer based on embryo morphology scores (36,37). The outstanding problem of this strategy is that it is impossible to establish a complete standard and quantitatively study embryo morphology, which may not be wholly consistent with its developmental potential. For this reason, it is of great significance to use embryonic fluid to establish a completely non-invasive, efficient, convenient, and sensitive detection method.

Distinct from the most classic MHC type Ia molecules, HLA-G5 belongs to the non-classical MHC complex Ib subtype. As an essential immune tolerance regulator in the human body, HLA-G5 is involved in processes such as foetal tolerance and autoimmunity during pregnancy, as well as inflammatory diseases and receiving allotransplantation from patients (37,38), and can achieve immune tolerance and protect the human body by interacting with inhibitory receptors on the surface of immune cells. HLA-G can interact with immunoglobulin-like proteins on dendritic cells (DC) (39,40), and through such self-made interactions, can suppress immune cells (41). Importantly, in normal tissues, HLA-G is most abundantly distributed on the surface of trophoblast cells in the placenta, which can effectively inhibit the local immune response in the uterus and promote the tolerance of the mother to the foetus (6,42). Based on this characteristic, the expression level of HLA-G is closely related to the success rate of embryo transfer during IVF treatment. In this study, an HLA-G aptamer was successfully prepared, which laid the foundation for detecting HLA-G expression in a minimal volume of embryonic fluid.

Biological macromolecules, including DNA, RNA, antibodies, and proteins, can be used as targets for aptamer detection (43). Traditional research mostly uses western blot, southern blot, or northern blot to detect biological macromolecules, and for soluble proteins in solution, ELISA is widely used (44,45). DNA aptamers are used for direct generation using a synthetic DNA library by the SELEX method. Aptamers are not only widely used in clinical testing, but their non-immunogenicity also provides them with significant therapeutic potential (46).

Aptamers with very high affinity to the target molecule help in early-stage diagnosis during disease progression, patient treatment, and the prevention of further disease spread. High-affinity aptamers are currently being tested in various clinical trials, and because of the high adaptability of chemically modified aptamers, stable aptamers have been produced and have been widely used in these trials (47). The preparation of HLA-G aptamers in this study is not only helpful for HLA-G-related research but also expands our broader understanding of aptamers. them

Traditional detection methods use chemiluminescence methods such as horseradish peroxidase (HRP), which has limited amplification of trace signals. Combining immunoassay technology with aptamers with golden nanoparticles or microfluidic chip technology, can build a portable and straightforward chip device to establish a fast, simple, and highly-sensitive detection method. Additionally, the radioisotopes (31P) can incorporate aptamer nucleic acid molecules to amplify the signal (48).

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-3334

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-3334

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-3334). The authors report funding support from the National Key Research and Development Program of China (No. 2018YFC1003100). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of the First Medical Center of Chinese PLA General Hospital (approval No.: s2019-196-01). The informed consent was waived due to the anonymous data and non-intervention feature of the study.

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