A novel test for annexin A5 M2 haplotyping in in vitro fertilization patients and preimplantation embryos

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Objective: To develop a test for evaluating the annexin A5 M2 haplotype in in vitro fertilization patients and preimplantation embryos.
Design: Test performance was measured by comparing Sanger sequencing of parental blood DNA and quantitative real-time polymerase chain reaction (qPCR) of saliva DNA, 3 fibroblast cell line 7-cell aliquots and their corresponding purified DNA, 123 trophectoderm biopsy samples, and DNA isolated from 1 embryonic stem cell line along with the Mendelian inheritance expectations, embryo Sanger sequencing, and single-nucleotide polymorphism (SNP) microarray-based linkage analysis.
Setting: Preimplantation genetic testing laboratory research on IVF patient and embryo DNA.
Patient(s): An assay was developed for the detection of the M2 haplotype on saliva samples of 6 in vitro fertilization patients. In addition, 13 patients who underwent preimplantation genetic testing with data on parental and embryo biopsy DNA available for research use were evaluated.
Intervention(s): None.
Main Outcome Measure(s): The concordance rates between Sanger sequencing, SNP array-based linkage analysis, and Mendelian inheritance expectations with qPCR.
Result(s): The concordance rate between Sanger sequencing and qPCR was 100% on parental blood DNA and saliva DNA. The sample concordance rate between all replicates of 7-cell aliquots was 100%. The sample concordance rate between 3 cell lines used to prepare 7-cell aliquots and purified genomic DNA was 100%. The concordance rate between qPCR and Sanger sequencing results from a single trophectoderm biopsy and isolated embryonic stem cell line was 100%. The concordance rate of trophectoderm biopsy qPCR results and expectations from Mendelian inheritance rules was 97%; however, when SNP array-based linkage analysis was included, the concordance rate reached 100%.
Conclusion(s): This study resulted in the development of a convenient saliva collection method and qPCR-based genotyping method to screen for the M2 haplotype. In addition, a novel method for testing preimplantation embryos has been established, providing an alternative to the use of low molecular weight heparin, through selection of embryos without the M2 haplotype. (Fertil Steril Sci® 2021;2:278–86. ©2021 by American Society for Reproductive Medicine.)
Key Words: Annexin A5, M2 haplotype, placental-mediated pregnancy complications, recurrent pregnancy loss, preimplantation genetic testing

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During the early stages of pregnancy, vascular remodeling between the placental villi and uterine wall is essential in establishing a circulatory system between the mother and fetus. The syncytiotrophoblasts of the placental tissue express annexin A5 (ANXA5) anticoagulant protein, which is essential in placental maintenance

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and repair (1, 2). ANXA5 aids syncytiotrophoblasts in maintaining placental vasculature and blood fluidity. A reduction in ANAX5 levels introduces the risk for thrombosis in the developing placenta and is associated with adverse pregnancy outcomes (3, 4). The adverse pregnancy outcomes originating from the placenta, known as placental-mediated pregnancy complications include recurrent pregnancy loss, stillbirth, preterm labor, and pre-eclampsia (5, 6). Preliminary data also show an association between M2 carriership and recurrent implantation failure (7). The association between M2 carrier status and risk for adverse pregnancy outcomes has been well established (8). Although the risk of negative impact is not absolute, a significant risk exists. There may be other unknown factors that can influence the severity of adverse pregnancy outcomes associated with M2 carriership. Four nucleotide substitutions in the core promoter of the *ANXA5* gene, known as the "M2" haplotype, decrease the levels of the ANXA5 protein in the placenta, increasing the risk of these pregnancy complications (9, 10). M2 variants decrease the ANXA5 protein expression levels. M1 variants have 40% less ANXA5 expression compared with normal haplotypes, and M2 variants have 60% less expression compared with normal haplotypes (11).

The M2 haplotype affects embryonic coagulation independent of inheritance from either the maternal or paternal genome and represents an important distinction from other known thrombophilias (12). In 44% of couples undergoing assisted reproductive technologies, one or both partners have been shown to carry the M2 haplotype (5). An intervention study using low molecular weight heparin (LMWH) has been conducted to counter the effects of the M2 haplotype, thus increasing the success rates from 16% to 42% (13). However, further evaluation is needed to determine the significant impact of LMWH treatment. LMWH treatment has been proven to be an effective strategy; however, directly testing preimplantation embryos from carrier patients can also be an effective alternative.

Given the negative association of the M2 haplotype in early pregnancy, early intervention may be warranted. In vitro fertilization (IVF) represents a logical setting for the evaluation of the clinical utility of M2 haplotype testing. The use of M2 carrier screening in the IVF population may help improve the outcomes in patients with a history of miscarriage, failed embryo transfers, or other pregnancy-related complications through personalized care and management.

Current available methods for M2 screening in adults involve extraction of DNA from peripheral blood and performing Sanger sequencing on purified DNA (5). The ability of Sanger sequencing to safely and cost-effectively test IVF patients and preimplantation embryos is limited. By contrast, the extraction of saliva-derived DNA and quantitative real-time polymerase chain reaction (qPCR) may provide an opportunity to improve the testing strategies in adults, with the latter method used to directly test preimplantation embryos for the first time. The present study aimed to develop and validate the ability to perform M2 testing on saliva DNA and preimplantation embryos using a variety of samples and molecular genetic approaches.

MATERIALS AND METHODS

Strategy

Conventional Sanger sequencing and a new qPCR-based assay were compared across several patient samples, including peripheral blood and saliva-derived DNA, to optimize the methodology and determine the concordance of M2 testing. The performance of qPCR on whole genome amplified DNA from limited numbers of cells, including cell lines and trophectoderm biopsy samples, was evaluated to determine the accuracy of preimplantation genetic testing (PGT) for the M2 haplotype.

Cell Lines and Genomic DNA

Cell lines and the corresponding purified genomic DNA were obtained from Coriell Institute (Camden, NJ). Fibroblast lines GM02637, GM02638, and GM02639 were cultured in accordance with the manufacturer's instructions. To model trophectoderm biopsy samples, 6–8 cells were loaded using an inverted microscope and 150-μm stripper tip (Cooper Surgical, Trumbull, CT) into 0.2-mL polymerase chain reaction tubes (USA Scientific, Ocala, FL). The samples were loaded using phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA). The purified genomic DNA (NA02637, NA02638, NA02639, NA02640, and NA02641) was quantified using a NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA).

Peripheral Blood

The DNA from peripheral blood was obtained using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Further processing was performed on 100 ng of extracted DNA as previously described (5).

Saliva

Saliva samples from patients undergoing clinical PGT to assess for monogenic disease, structural rearrangements, and polygenic disease (PGT-M, PGT-SR, and PGT-P) (Genomic Prediction Clinical Laboratories, North Brunswick, NJ) were utilized. All patients provided an informed consent to have their samples be used for research purposes. Saliva samples were collected using the ORA-Collect Dx kit in accordance with the manufacturer's instructions (DNA Genotek, Inc., Ottawa, Ontario, Canada). The samples were stored at room temperature for a maximum of 7 days before further processing.

Trophectoderm Biopsy Samples

Aliquots of whole genome amplified DNA from 123 trophectoderm biopsy samples from 13 couples were obtained after the completion of clinical PGT (Genomic Prediction Clinical Laboratory, North Brunswick, NJ).

Embryonic Stem Cells

The cells from 1 isolated embryo were used to create an embryonic stem cell (ESC) line, eysES4, derived as previously described (14). The cells underwent whole genome

application, and DNA was further processed. The samples were diluted to 10 ng/μL and stored at −20°C.

DNA Extraction

Before extraction, the saliva samples were incubated at 50°C in a VWR Forced Air Oven (VWR, Radnor, PA) overnight. After an overnight incubation, the DNA was extracted from 200 μL of saliva using the Qiagen EZ1 Advanced XL system and the EZ1 DNA tissue kit following the EZ1 Advanced XL protocol as recommended (Qiagen, Germantown, MD). The extracted DNA was quantified using the NanoDrop 8000. The samples were diluted to 20 ng/μL and stored at −20°C.

Whole Genome Amplification

Embryo biopsy samples and 7-cell aliquots from fibroblast cell lines were amplified as previously described (15). Amplification was performed using Applied Biosystems 2720 Thermal cycler (Thermo Fisher Scientific, Waltham, MA). After completion, the samples were quantified using the NanoDrop 8000. The samples were diluted to 10 ng/μL.

Preamplification

Preamplification was performed with primers outside the loci of 4 TaqMan allelic discrimination assays utilizing the AccuPrime GC Rich DNA Polymerase kit (Thermo Fisher Scientific, Waltham, MA) using 1 μL of DNA (5–100 ng/μL) following the recommended protocol. The samples were stored at −20°C until further processing.

Sanger Sequencing

Sanger sequencing was performed at GeneWiz (South Plainfield, NJ). The procedure was performed by visual analysis of each of the 4 single-nucleotide polymorphisms (SNPs) using a SnapGene analysis viewer software.

Taqman Assay Design and qPCR

Custom taqman assays were designed and ordered from Thermo Fisher Scientific (Waltham, MA) for each of the 4 mutation sites. Preamplification was performed with primers outside the loci of the 4 TaqMan assays. Allelic discrimination was performed using 2 μL of the preamplified sample in a 10-μL reaction volume with Genotyping Master Mix and a QuantStudio 3 system (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's recommendations.

SNP Array

SNP array linkage analysis was performed on samples that were processed through the Gene Titan system (Affymetrix Inc, Santa Clara, CA). Sample preparation and linkage analysis were performed as previously described (15).

Concordance Analyses

Results of qPCR-based genotyping methods were compared with those of conventional Sanger sequencing methods, ESC DNA analysis, and SNP linkage analysis. Concordance was confirmed when the observed results matched with those expected.

Institutional Review Board Approval

Cell lines and genomic DNA obtained from Corriell Institute were utilized in accordance with their materials and transfer agreement (assurance form). Patient samples, including saliva and embryo biopsy samples, were utilized from patients who consented to donate samples to research for training and validation purposes, quality control, research, and publications. All samples were deidentified, and the patients were not notified of the results on research completion. The requirement for the reanalysis of data obtained from clinical genetic testing was waived by the Western Institutional Review Board (exemption WIRB work order: 1-327506-1). The material evaluated in this portion of the research was exempted from further review by the Western Institutional Review Board. Evaluation of a trophectoderm biopsy sample and ESCs from a single embryo was performed with approval from Columbia University's Embryonic Stem Cell Committee and institutional review board and informed patient consent.

RESULTS

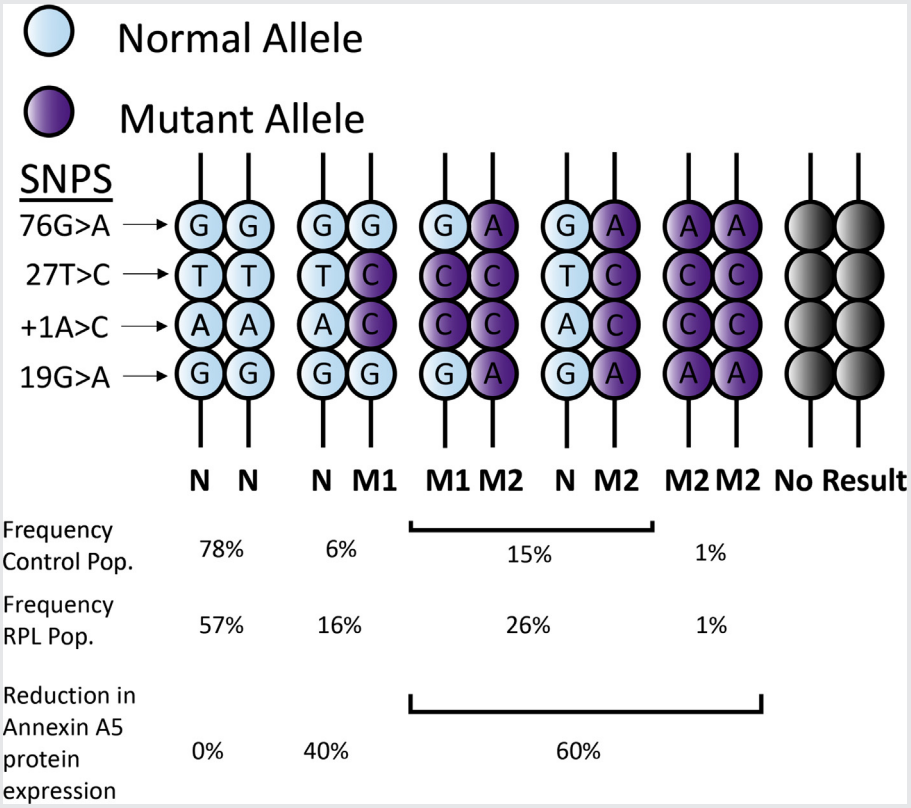
Saliva M2 qPCR Validation

Conventional TaqMan genotyping on DNA extracted from patient samples initially resulted in failure to produce accurate M2 haplotypes, as only 3 of the 4 assays produced interpretable allelic discrimination data. To optimize the results, a preamplification method was developed to amplify the target region before qPCR. Sanger sequencing confirmed the alignment to the human genome using the basic local alignment search tool specific to nucleotide sequences (BLASTN). With the performance of preamplification before qPCR, interpretable allelic discrimination data were produced for all 4 SNPs, and M2 haplotypes were detected (Fig. 1). Previous M2 haplotype screening methods (5) utilized DNA derived from patient blood samples. The samples had been previously tested using conventional M2 haplotyping methods. A total of 16 blood-derived patient samples were subjected to qPCR. Fifteen of the 16 samples were concordant when comparing qPCR developed in this study, and Sanger sequencing obtained from the original laboratory. To perform a third analysis for confirmation, the samples were evaluated by a third laboratory using Sanger sequencing. All 16 (100%) of the qPCR-based results were confirmed. Finally, saliva-derived DNA was evaluated by qPCR and Sanger sequencing and resulted in 100% concordance (n = 6) (Fig. 2). In addition to the 16 blood samples and 6 saliva samples, 16 additional samples, which included purified genomic DNA from cell lines and additional genomic DNA from the subjects, were evaluated, demonstrating a 100% concordance between qPCR and Sanger sequencing, confirming the validity of the qPCR-derived assay.

PGT for M2

To model the amount of DNA obtained from a typical trophectoderm biopsy sample, 7-cell aliquots of 3 fibroblast cell lines

FIGURE 1



A schematic representation showing the alleles of different M2 genotypes. Each allele shows the presence of the normal or mutated alleles of the single-nucleotide polymorphisms that make up the M2 haplotype. Normal alleles are presented in blue, and mutant alleles are presented in purple. Each SNP associated with the M2 haplotype is represented by a *circle*. Listed below are the observed frequencies of the haplotype in a control population as well as a recurrent pregnancy loss population (5). Reduction in annexin A5 protein expression for each genotypic combination is also listed (11). SNP = single-nucleotide polymorphism.

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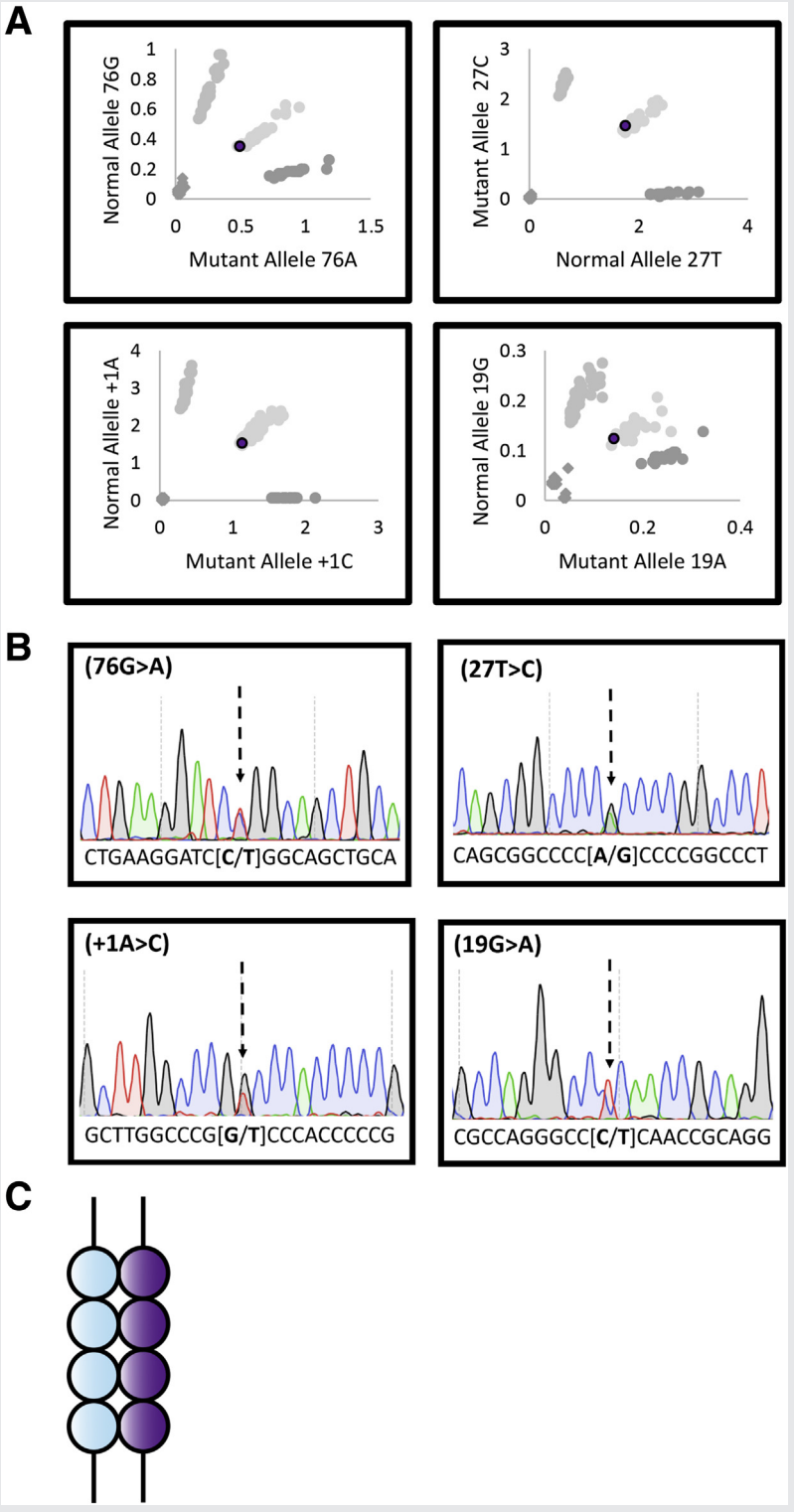
(limited quantities) were evaluated by whole genome amplification and qPCR analysis (16). Meanwhile, the corresponding purified genomic DNA (bulk quantities) was evaluated by conducted qPCR analyses only. Each cell line sample was run in biologic replicates of 8 to evaluate reproducibility. The sample concordance between replicates of each cell line was 100%. Results of the cell aliquots for each cell line were compared with those of the corresponding purified DNA, and the concordance rates were 100%. Preamplification products of fibroblast cell lines and purified genomic DNA were also analyzed by Sanger sequencing, which confirmed all the qPCR results (100%) (Fig. 3). Parental DNA of cell lines were also run to confirm their consistency with Mendelian inheritance patterns.

Several methods were used to evaluate the validity of results from trophectoderm biopsies. First, the DNA from both trophectoderm biopsy samples and an ESC line derived from the same embryo were compared and showed concordance (Fig. 4 and Supplemental Fig. 1 [available online]) (14). ESCs and DNA derived from the same embryo were examined to test the limited quantities of DNA from an embryo biopsy sample (a clinically relevant sample source) along with the large quantities of DNA from the same embryo. Second, several trios (n = 13) were evaluated for concordance with Mendelian rules of inheritance. Saliva-derived parental DNA and trophectoderm whole genome amplified DNA were analyzed by qPCR. SNP array data from each trio were also used to evaluate the linkage-based prediction of the M2 haplotype. The trophectoderm biopsy qPCR results were 97% (120/123) concordant with the expectations from Mendelian inheritance rules and 100% (123/123) when including SNP array-based linkage analyses (Fig. 5 and Supplemental Fig. 2 [available online]). When 1 parent was a carrier, 46% (39/84) of the embryos were M2 carriers. In families where both parents were carriers, 91% (20/22) of embryos were M2 carriers. The trophectoderm qPCR allele dropout rate was 5% (n = 116) when compared with linkage-based analysis. Of the 123 embryos used for this analysis, qPCR results were obtained from 117 embryos. Results of linkage-based analysis were obtained from 118 embryos. The samples used in both platforms failed to produce a result because of the low amplification.

FIGURE 2



(**A**) Allelic discrimination plots for each of the 4 single-nucleotide polymorphisms of the M2 haplotype are shown for a heterozygous M2 carrier from DNA derived from a saliva sample. (**B**) Sanger sequencing results of a heterozygous M2 carrier for each of the 4 single-nucleotide polymorphisms are shown. The *arrows* highlight the presence of both alleles at the specific single-nucleotide polymorphism location. (**C**) Shown is a schematic representation of the chromosomes and alleles present in an M2 heterozygous carrier.

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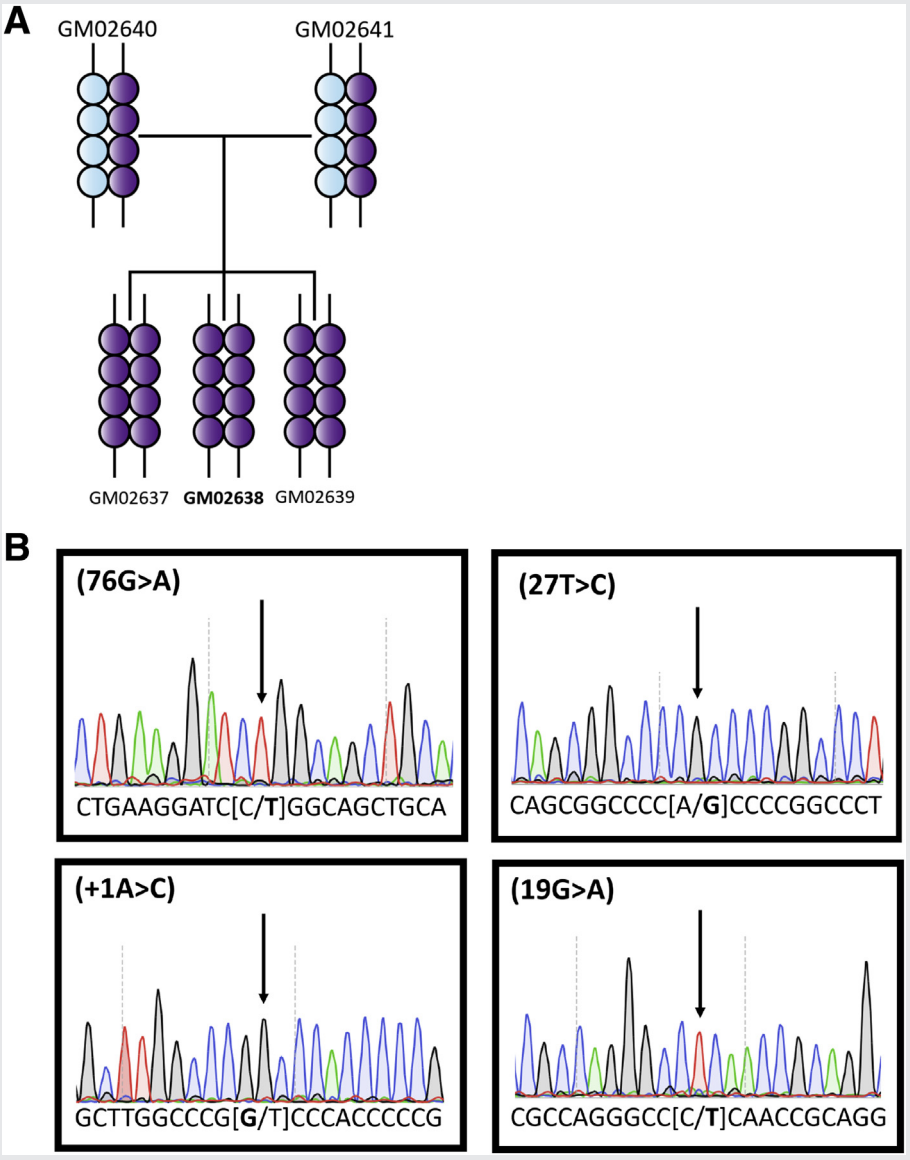
FIGURE 3



(**A**) A family pedigree outlines the M2 carrier status in parents and offspring of cell lines obtained from the Coriell Cell Repository. A heterozygous carrier couple (GM02640 and GM02641) pass down mutant M2 alleles, which make the offspring M2 homozygous carriers (GM02637, GM02638, and GM02639). (**B**) Sanger sequencing results of GM02638 carrier offspring are shown. The *arrows* point to the homozygous presence of the mutant alleles inherited.

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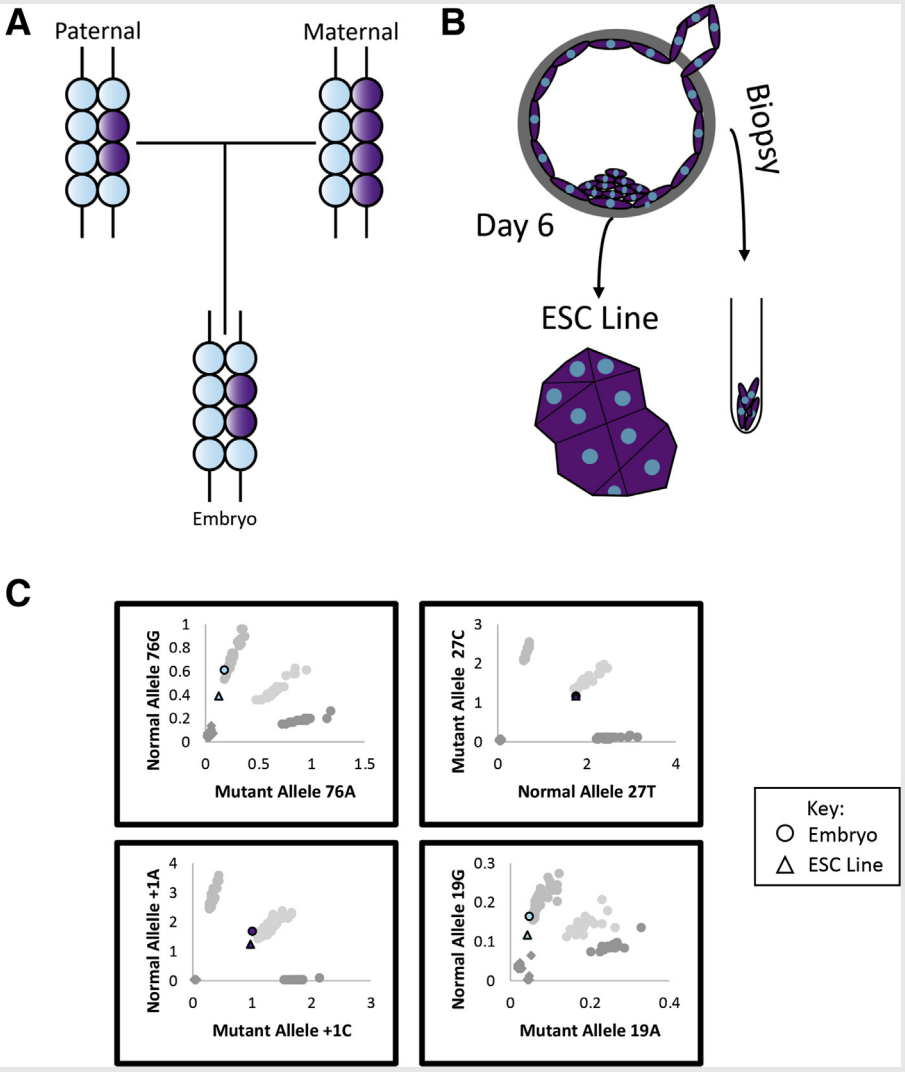
DISCUSSION

This novel test included the extensive validation of fibroblast cell lines, trophectoderm biopsies, trio analyses, analysis of ESC lines, and SNP array linkage-based analysis. The concordance rate for samples derived from parental blood and saliva for the newly developed M2 haplotype testing and conventional testing methods involving Sanger was 100%. To test the accuracy of the qPCR-derived M2 haplotype test for embryos, familial case analyses including multiple different sample types were utilized, and the results were compared with the corresponding genomic DNA analysis, ESC DNA analysis, and Sanger sequencing results and Mendelian inheritance rules. Our results matched with those expected, with a concordance rate of 97%–100%.

The sample collection method used for this novel M2 test was more convenient than the conventional intravenous blood collection method without compromising the genotyping results (17). The qPCR-based methodology is also less expensive and faster than the standard Sanger sequencing methods. These factors would provide an opportunity to broaden its utilization. Several studies on the association of the M2 haplotype with recurrent pregnancy loss have been completed (1, 5, 6, 10, 11,

FIGURE 4



(**A**) A family pedigree outlines the M2 carrier status in parents and offspring. A heterozygous carrier mother transmits a normal allele, and an M1 carrier father transmits an M1 allele, which made the offspring an M1 carrier. (**B**) A schematic representation showing a generalized process of obtaining an ESC line and a trophectoderm biopsy from a blastocyst. (**C**) Allelic discrimination plots compare the results of an embryo trophectoderm biopsy with isolated ESC DNA for each of the 4 alleles that make up the M2 haplotype. ESC = embryonic stem cell.

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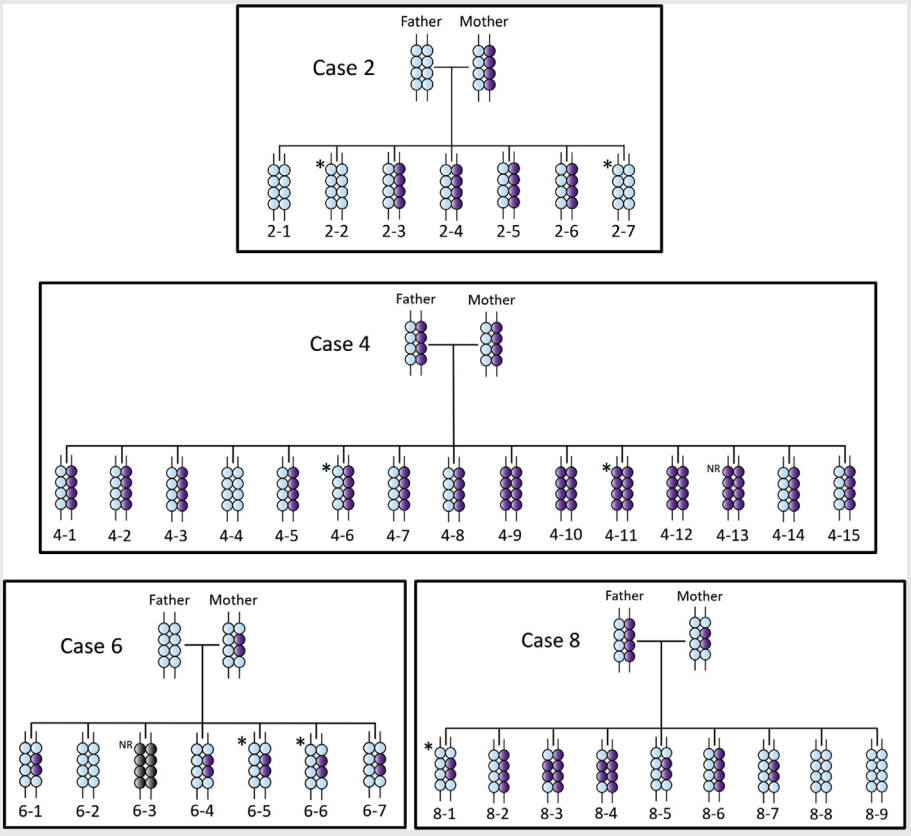
18, 19). These studies have included analysis of individuals from the United Kingdom, Estonia, Italy, Germany, Japan, and Argentina (1, 5, 6, 10, 11, 18, 19). However, very few studies have specifically evaluated patients undergoing infertility treatment (5). In addition, most of these studies evaluated the maternal genotypes only, without knowledge of the paternal contribution or the genotype of the fetus itself (8). The development and validation of the PGT method will allow further evaluation of the significance of embryonic M2 carriers in a clinical setting. The application of this method in the IVF setting may allow the collection of these additional data, which could provide broader opportunity for early intervention.

Intended parents with a history of placental-mediated pregnancy complications such as recurrent pregnancy loss, preterm labor, pre-eclampsia, small for gestational age infants, and venous thromboembolism may benefit from M2 haplotype testing. Patients who are carriers of the M2 haplotype currently have the option of utilizing the LMWH treatment. However, preliminary data suggest that adverse outcomes are associated with embryonic rather than parental haplotypes. Although testing the intended parents can provide useful information on the risk of transmission, directly evaluating the embryo may be of more value. In couples where only 1 parent is a carrier, there is only a 50% chance that the embryo will also be a carrier. In couples were both parents are carriers, there is a 75% chance that the embryo will be a carrier. In these scenarios, there is still a chance that the embryo might not be a carrier of the M2 haplotype, in which case it may not exhibit any resultant unfavorable pregnancy outcomes. Although the female carrier may still

FIGURE 5



Pedigree analyses of selected familial cases (trios) that were tested to show the Mendelian inheritance patterns of the M2 haplotype. Parents and embryos were individually tested, and the results were compared for concordance with possible inheritance patterns. *Asterisks* indicate aneuploid embryos; superscript NR denotes that no karyotype data were obtained.

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be administered with LMWH as a pre-emptive treatment option, in many cases, it would be unnecessary. One of the objectives of the present study was to develop a novel M2 screening platform capable of accurately genotyping limited quantities of DNA obtained from an embryo biopsy sample. This provides a new option (PGT-M for M2) for carrier couples to consider as an alternative to universal LMWH treatment. In many cases, patients may produce euploid, M2-free embryos, mitigating the need for LMWH injections.

The limitations of utilizing qPCR for PGT-M include allele dropout because of amplification bias. To measure the allele dropout rates of this platform, samples evaluated by qPCR were analyzed using SNP array linkage analysis, and the results of both platforms were analyzed. The parental and embryo samples were evaluated using the Affymetrix Gene Titan microarray system. A total of 123 trophectoderm biopsy samples, in which none of the parents were carriers, at least 1 parent was a carrier, or both parents were carriers of the M2 variant, were screened. Parental data can be used to predict embryo carriership using linked markers. Linkage analysis determined that the qPCR M2 testing platform performed with a 5% allele dropout rate. However, embryonic allele dropout was mitigated by combining SNP array-based linkage analysis with direct qPCR-based testing of the M2 haplotype. This is a common approach in PGT-M, which is also utilized when evaluating the M2 haplotype.

Future clinical research studies will further investigate the association between the history of pregnancy complications and M2 carrier status, specifically in IVF patients, and evaluate the utility of M2 haplotype PGT in embryos from carrier couples. This trial may also shed further light on the efficacy of LMWH treatment. With the use of M2 haplotype PGT, success rates of both treatment options can be compared. Carrier couples who underwent LMWH therapy may have improved pregnancy outcomes (13). However, there are currently no data involving the characterization of embryo carrier status in pregnancies of carrier couples. Therefore, the observed benefit of LMWH treatment in carrier couples may be dampened in cases where the embryo itself is not a carrier of the M2 haplotype. The use of PGT-M for the detection of the M2 haplotype may provide an opportunity to develop an effective targeted intervention and to conduct additional investigations.

CONCLUSION

This novel M2 test is a new tool to further investigate the association of embryonic M2 carrier status with pregnancy complications after IVF. Testing of saliva samples and fast results may enable more efficient, patient-friendly integration of clinical care. Patients experiencing recurrent pregnancy loss may benefit from M2 carrier screening. Intended parents found to be carriers of the M2 haplotype have the option of undergoing the LMWH treatment. As a result of the present study, IVF couples may also elect to undergo PGT-M for the detection of the M2 haplotype on their preimplantation embryos, to enable the development of a more targeted approach. Future trials are ongoing to evaluate the potential benefits of this testing and its associated interventions.

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