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ORIGINAL RESEARCH

Application of Reproductive Related Gene Detection in Etiological Analysis of Recurrent Non-Transplantable Embryo

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Abstract

Objective: Gamete maturation and embryonic development are directly related to genetic factors. If the patient has genetic abnormalities, the efficacy of clinical treatment may be seriously affected or even ineffective. In this study, we analyzed the variation of reproductive related gene associated with the adverse outcome of assisted reproductive therapy, recurrent non transferable embryo, and discussed the key points of genetic counseling and the selection of clinical treatment schemes for recurrent non transferable embryo patients.

Methods: This study retrospectively analyzed 44 infertile females, 13 azoospermic males of repeated embryonic development block, analyzed the results of gene testing, assisted reproduction scheme and outcome.

Results: Abnormal gene variation was detected in 24 cases of the 44 female who participated in this study, the abnormal rate was 54.55%. ZP2, GDF9, ERCC6 and IL17RD were found to have higher mutation frequencies. There were 13 male patients with typical infertility, including 9 cases with azoospermia, 3 cases with oligospermia and 1 case with severe oligospermia. Abnormal gene variation was detected in 12 cases, and the abnormal rate was 92.31%.

Conclusion: According to the advice of clinicians and embryology experts, the gene detection of women and/or men can be targeted, which is helpful to find the cause of recurrent non transferable embryo and avoid unnecessary waste of medical resources.

Keywords

Infertility, Recurrent non-transplantable embryos, Genetic characteristics, Gene sequencing, Genetic variation

Introduction

In vitro fertilization and embryo transfer (IVF-ET) and its derivative technology have undoubtedly brought hope to many infertile couples all over the world. However, among infertile patients treated by various assisted reproductive methods, the average pregnancy rate is only 34%, while the failure rate is about 60% [1]. It is a painful experience to both wife and husband, for no transferable embryo after multiple IVF cycles. And which undoubtedly has negative physical and psychological effects. Studies have found that about 30% of male infertility and 10% of female infertility are caused by genetic reasons [2]. NGS panel is one of the most effective methods to study infertility related genes and find complex hereditary pathogenic genes [3]. At present, there are few reports on the research of infertility related gene NGS panel on the etiology of repeated multiple IVF cycles non-transplantable embryos.

Therefore, the purpose of this study is to explore the application of reproduction related gene detection in the etiological analysis of repeated non-transplanted embryos. For couples with recurrent non-transplantable embryos, use a specific NGS panel to analyze one or both reproductive related genes to find the mutation sites of pathogenic genes. Combined with their gene phenotype, clinical indications and prognosis, the clinical significance and application of reproductive related gene detection were discussed.

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Research Object and Method

General information

The study collected patients presented between 2018-2020 in Hebei Institute of Reproductive Health Science and Technology and Hebei Reproductive Hospital to receive IVF/ICSI assisted pregnancy treatment again. Inclusion criteria: 1) Age 20~42 years-old; 2) Primary sterility and infertility; 3) At least two IVF/ICSI with non-transplantable embryos occurred in the past. Exclusion criteria: 1) Chromosome G banding analysis showed abnormality and polymorphism; 2) Received bone marrow transplantation and other organ transplantation; 3) Received chemotherapy and radiotherapy; 4) Immune-related diseases and long-term use of immunosuppressive agents; 5) Patients with previous history of trophoblastic diseases and malignant tumors. After analyzing the possible causes of previous non transplantable embryo cycle by clinicians and embryologists, all the selected persons were evaluated to have different degrees of reproductive dysfunction or germ cell development defects, such as oocyte maturation disorder, ovarian dysfunction, spermatogenesis disorder, severe sperm morphological abnormality (round-headed spermatozoa), embryo development block and so on. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Hebei Institute of reproductive health science and technology (No. A202002012) and informed consent was taken from all the patients.

Specimen collection and treatment

After inclusion and exclusion criteria were applied, 6 mL of peripheral blood was collected from patients and placed in the blood collection vessel contained EDTA-K2 anticoagulant. Using the DNA Extraction Kit (Qiagen, Germany), according to the kit instructions, genomic DNA was isolated from peripheral blood leukocytes for NGS panel sequencing.

Screening of candidate pathogenic gene mutations

By screening reproductive related candidate genes, two groups of NGS panel developed according to their pathogenicity include 79 female reproductive related genes; 116 genes and Y chromosome microdeletion sites in male. The gene information contained in Panel is shown in Table 1a and Table 1b, including the known reproduction related single genes and non-syndrome genes. After the analysis of the sequencing data, the mutation sites of suspected pathogenic genes were obtained. Gene sequencing was completed by Shanghai Yikon Medical Laboratory Co., Ltd.

Results

Genetic testing and analysis of female

44 females were tested in 57 couples who recurrent non-transplantable embryos. Among 44 females, 24 cases had abnormal gene variation, 20 cases normal, the abnormal rate was 54.55%. The genes with high mutation frequency were found to be ZP2, GDF9, ERCC6 and IL17RD. The Mutant frequency and corresponding clinical symptoms of abnormal variation are shown in Table 2. Among them, 14 cases with single gene mutation, 6 cases with two gene mutations and 4 cases with three or more gene mutations. In these cases, KAL1 c.1678g > A and WEE2 c.1576t > G were considered hot spot mutations (Table 2). Four patients with ZP2 and/or ZP3 gene mutations showed abnormal fertilization. Corresponding to the clinical symptoms of patients, we found that gene variation was directly related to clinical phenotype. Clinical manifestations of a female with both WEE2 and TLE6 gene mutations: Initially, there were 30 antral follicles in two ovaries, 5 dominant follicles developed and 4 mature oocytes were obtained after two routine treatments (excluding iatrogenic low response caused by insufficient treatments), but there are no transplantable embryos after artificial insemination. The doctor chose flexible antagonist protocol to promoting ovulation, while, the use of antagonists was abandoned because the LH level did not reach 4 IU/L in the whole process of ovulation induction. A total of 54 oocytes were obtained from the twice oocytes retrival operations, but all of them were Metaphase I (MI). After culture *in vitro*, the oocytes still cannot develop and mature to the metaphase of second meiosis, cannot undergo ICSI operation and development embryos.

Table 1a: Female gene panel information.

Detection item	Detection gene
Premature ovarian failure related genes (35)	PSMC3IP, FSHR, NOBOX, BMP15, FIGLA, FMR1, ERCC6, FOXL2, HFM1, MCM8, POF1B, STAG3, NR5A1, SYCE1, EIF2B2, EIF2B4, EIF2B5, MSH5, MCM9, SOHLH1, CLPP, ERAL1, HSD17B4, AMH, SOX9, DIAPH2, LMNA, FANCM, POF1B, GDF9, AARS2, ESR2, MRPS22, NUP107, BRCA2
Genes related to oocyte maturation defects, fertilization and embryo development abnormalities (11)	TUBB8, ZP1, PATL2, ZP3, ZP2, PADI6, WEE2, TLE6, ZP4, KHDC3L, PANX1
Genes related to idiopathic hypogonadotropic hypogonadism (33)	TACR3, TAC3, WDR11, KISS1, KISS1R, SPRY4, IL17RD, FEZF1, FGF17, FLRT3, GNRH1, GNRHR, DUSP6, LHB, FGFR1, PROKR2, FGF8, PROK2, CHD7, ANOS1, LHCGR, POR, HS6ST1, SEMA3A, NSMF, NR0B1, PNPLA6, SMCHD1, POLR3B, POLR3A, RNF216, SOX2, SOX10

Table 1b: Male gene panel information.

Detection item	Detection gene
Genes related to congenital absence of vas deferens (2)	CFTR, ADGRG2
Spermatogenesis disorder related genes (38)	KLHL10, CATSPER1, NANOS1, SLC26A8, NR5A1, SYCE1, SYCP3, TAF4B, TEX11, USP9Y, SEPTIN12, TEX15, CEP19, CFAP43, CFAP44, BRDT, AK7, CFAP69, TSGA10, PLCZ1, MEIOB, TEX14, NOS3, H2BFWT, RHOXF1, RHOXF2, RHOXF2B, OGG1, XRCC1, TRICH9, QRICH2, ZMYND15, PMFBP1, FSIP2, FANCM, SPINK2, WDR66, SRY
Y chromosome microdeletion (1)	AZF
Non-syndromic polymorphism abnormal of sperm flagella (1)	DNAH1
Headless sperm (1)	SUN5
Genes related to roundhead spermatozoa (3)	AURKC, DPY19L2, SPATA16
Genes related to primary ciliary dyskinesia (36)	LRRC6, DNAI2, RSPH1, DNAAF2, CCDC114, ZMYND10, ARMC4, CCDC151, CCDC103, SPAG1, DNAAF1, RSPH4A, DNAH5, DNAI1, CCDC40, CCDC39, DNAH11, CCNO, DNAAF3, DNAAF4, DNAJB13, DNAL1, DRC1, GAS8, CFAP298, CCDC65, RSPH3, RSPH9, TTC25, PIH1D3, NME8, HYDIN, DNAH9, DNAAF5, CFAP300, LRRC56
Cryptorchidism-related genes (1)	INSL3
Genes related to androgen insensitivity syndrome (1)	AR
Genes related to idiopathic hypogonadotropic hypogonadism (33)	TACR3, TAC3, WDR11, KISS1, KISS1R, SPRY4, IL17RD, FEZF1, FGF17, FLRT3, GNRH1, GNRHR, DUSP6, LHB, FGFR1, PROKR2, FGF8, PROK2, CHD7, ANOS1, LHCGR, POR, HS6ST1, SEMA3A, NSMF, NR0B1, PNPLA6, SMCHD1, POLR3B, POLR3A, RNF216, SOX2, SOX10

Table 2: Statistics of female NGS panel results.

Mutant gene	Mutation frequency	Related diseases	Genetic model
ZP2	4	Oocyte maturation defect	AR
TUBB8	2	Oocyte maturation defect	AD/AR
WEE2	2	Oocyte maturation defect	AR
ZP3	1	Oocyte maturation defect	AD
PANX1	1	Oocyte maturation defect type 7	AD
IL17RD	3	Hypogonadotropic hypogonadism	AD/AR
PROKR2	2	Hypogonadotropic hypogonadism	AD
KAL1	2	Hypogonadotropic hypogonadism	XLR
SPRY4	1	Hypogonadotropic hypogonadism	AD
GNRHR	1	Hypogonadotropic hypogonadism	AR
HS6ST1	1	Hypogonadotropic hypogonadism type 15 with or without anosmia	AD
GDF9	4	Premature ovarian failure	AR
ERCC6	3	Premature ovarian failure	AD
HFM1	1	Premature ovarian failure	AR
EIF2B2	1	Premature ovarian failure	AR
FMR1	1	Premature ovarian failure	XL
DIAPH2	1	Premature ovarian failure	XLD
PSMC3IP	1	Ovarian dysgenesis	AR
MCM9	1	Ovarian dysgenesis	AR
NUP 107	1	Ovarian dysgenesis type 6	AR
POR	2	Cytochrome P450 oxidoreductase deficiency	AR
TLE6	1	Preimplantation embryonic lethality	AR
PADI6	1	Preimplantation embryonic lethality	AR
LHCGR	1	Luteinizing hormone resistance	AR
BMP15	1	Premature ovarian failure 4/Ovarian dysgenesis 2	XL

AD: Autosomal Dominance; AR: Autosomal Recessive; XLD: X Linked Dominance; XLR: X-Linked Recessive

Table 3: Statistics of male NGS panel results.

Mutant gene	Mutation frequency	Related diseases	Genetic model
DNAH1	13	Primary ciliary dyskinesia, Spermatogenic failure	AR
DNAH9	5	Primary ciliary dyskinesia	AR
NME8	4	Primary ciliary dyskinesia	AR
DNAH5	1	Primary ciliary dyskinesia	AR
CCDC 40	1	Primary ciliary dyskinesia	AR
DNAI2	1	Primary ciliary dyskinesia	AR
FSIP2	4	Spermatogenesis disorder	AR
TEX15	4	Spermatogenesis disorder	AR
AK7	2	Spermatogenesis disorder	AR
USP9Y	1	Spermatogenesis disorder	YL
CFAP43	1	Spermatogenesis disorder	AR
SOHLH1	1	Spermatogenesis disorder	AD
KLHL10	1	Spermatogenesis disorder	AD
SUN5	1	Spermatogenesis disorder	AR
KAL1	1	Hypogonadotropic hypogonadism	XLR
TACR3	1	Hypogonadotropic hypogonadism	AR
POR	1	Cytochrome P450 oxidoreductase deficiency	AR
DPY19L2	2	Round-headed spermatozoa, Spermatogenesis disorder	AR
AZFc deletion	1	Azoospermia or oligospermia	

AD: Autosomal Dominance; AR: Autosomal Recessive; XLD: X Linked Dominance; XLR: X-Linked Recessive; YL: Y Linked

Genetic testing and analysis of male

13 males were tested in 57 couples who recurrent non-transplantable embryos. Their clinical manifestations: 9 cases were azoospermia, 3 cases were oligospermia (including 1 case of round-headed spermatozoa) and 1 was severe oligospermia. Abnormal gene variation was detected in 12 cases, and the abnormal rate was 92.31%. All male patients underwent preset NGS panel sequencing (Supplementary Table 1 and Supplementary Table 2 for detailed results). According to the statistics of NGS panel results (Table 3), the genes with high mutation frequency were DNAH1, DNAH9, NME8, FSIP2 and TEX15. Except for one patient who only carried DPY19L2 homozygous mutation, all the other male patients carried two or more variants. DNAH1, DNAH9 and TEX15, which are prone to mutation, are closely related to spermatogenesis disorder and primary ciliary dyskinesia.

Discussion

Genetic factors play an important role in the occurrence of infertility, such as chromosome translocation, inversion and genome copy number variation [4]. These genetic variations seriously affect gamete formation, fertilization and normal development of oosperm. Studies have found that about 30% of male and 10% of female infertility are caused by genetic reasons [2]. This study also found that the incidence of gene abnormality in female patients was 54.55%, and that in male patients was 92.31%, which was higher than the data reported in other studies. This is related to the common clinical symptoms of the cases in this study: recurrent non transplantable embryo in IVF/ICSI cycles, all cases were assessed to have different degrees of reproductive dysfunction or germ cell development defects through the analysis of the causes of previous non transplantable embryo cycles by clinicians and embryologists.

It has been found that the accurate and orderly transcription and expression of some specific key genes play a vital role in the development and normal function of reproductive system. For example, germ cell transcription factors such as WEE2 [5] and SOHLH2 [6] can form complex specific transcription regulatory networks, regulate the expression of downstream related genes and maintain the normal function of reproductive system. In this study, a female with both WEE2 and TLE6 gene mutations showed 30 antral follicles in two ovaries, 5 dominant follicles developed after two routine treatments (excluding iatrogenic low response caused by insufficient treatments), 4 mature follicles were obtained, but no embryos could be transferred. It is consistent with the symptoms reported in previous studies. The zona pellucid (ZP) gene regulates the formation and maturation of zona pellucid, ZP variation may lead to failure of normal fertilization due to zona pellucida deletion or abnormal morphology [7]. Some cases with the similar symptomatic existed in our study, 4 female patients with ZP2 and/or ZP3 mutations showed abnormal fertilization. NAH1, DNAH9 and TEX15 have higher mutation frequency in male. Among them, the clinical manifestation of 1 male patient was

severe oligoasthenospermia who with the pathogenic variation of DNAH1 gene. No abnormality was found in the mature follicles of his spouse after embryologist evaluation, but the low fertilization rate after ICSI and there were no transplantable embryos finally. The generation of germ cells is interacted and coordinated by various signal pathways. For specific patients, the NGS panel of infertility related genes have a great clinical significance to find out the causes of repeated non-transplantable embryos.

This study found that there are 25 and 18 genes with high mutation frequency in female and male. We found some hot spot mutations also, such as KAL1 c.1678G > A and WEE2 c.1576T > G in female, which are related to X-linked recessive hypogonadotropic hypogonadism and autosomal recessive oocyte maturation defect respectively. DNAH1, DNAH9, NME8 and TEX15, which are prone to variation in male, are related to spermatogenesis disorder and primary ciliary dyskinesia closely. NME8 c.793G > T and c.650T > C, which gene hot spot mutations in male, are associated with autosomal recessive primary ciliary dyskinesia. All these results indicate that reproductive genes play an important role in the production of germ cells and embryonic development.

At present, dozens of gene variations related to reproductive development have been found, which can lead to primary infertility, and some infertile patients carry two or more gene variants [7]. This study showed that among 20 female patients with reproductive related gene variations, 6 patients carried two gene variations and 4 patients carried three or more gene variations, the male patients with reproductive related gene variations, except 1 case with DPY19L2 homozygous mutation, all the other patients carried more than two variants, which is basically consistent with previous related studies.

After multiple IVF/ICSI cycles without transplantable embryos, patients will not only waste time and money, but also cause great psychological pressure, if the routine treatment is continued and the cause is not found through genetic testing. Therefore, patients before entering the assisted pregnancy cycle, reproductive related gene testing to identify possible genetic factors can reduce the risk of assisted pregnancy failure. In addition, genetic testing may also be conducive to the selection of appropriate and efficient drugs or treatments. For example, patients with sperm dysfunction caused by TTC29 variation, traditional antioxidant treatment schemes are ineffective, the treatment scheme of ICSI can be recommended directly. It not only saves the waste of time and money caused by long-term drug treatment, but also achieves satisfactory pregnancy outcome faster [8]. Moreover, genetic testing can judge the prognosis of microsurgical sperm extraction in patients with non obstructive azoospermia (NOA). In NOA patients with AZFa or AZFb deletion, it is difficult to obtain sperm through microsurgical sperm extraction [9], Tex11 and MEIOB mutants can lead to meiotic arrest and NOA, and their postoperative effect is also unsatisfactory [10]. Gene testing can identify the source of gene variation and suggest the disease risk of other members in the family, so as to detect and intervene as soon as possible and avoid wasting reproductive time.

Although NGS panel can detect multiple pathogenic gene mutations at the same time, but its detection range is limited because gene mutations outside the preset panel cannot be detected. Plus, due to insufficient relevant database evidence, lack of relevant report, or the clinical significance of relevant reports is disunity, which has a great impact on the interpretation of some gene variants, the variation rating with unclear clinical significance has been obtained. All these will cause a certain degree of trouble to clinicians in genetic counseling. With the increasing and deepening of reproductive gene research, more gene functions will be confirmed and the database will be enriched, which will greatly improve this puzzle.

To summarise, For patients with primary infertility who suffered recurrent non-transplantable embryos, the reproductive genetic testing can analyze the possible causes according to clinicians and embryology experts, which is not only helpful to find the cause of the disease, but also avoid unnecessary waste of medical resources and reduce the economic burden of patients and society.

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Author Contributions

L Zhang and YJ Zhou conceived and designed the study; P Liu, Y Geng collected the data; P Liu and L Zhang analyzed the data and drafted the manuscript; P Liu and Y Geng performed the experiments; L Zhang and YJ Zhou assisted with data analysis. All authors have read and approved the manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest statement

Authors declare no conflict of interest with respect to the authorship and/or publication of this article.

Ethics approval and consent to participate

The study was approved by ethics board of Hebei Institute of reproductive health science and technology and informed consent was taken from all the patients.

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