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EXPERIMENTAL STUDY

Preimplantation genetic diagnosis of X-linked diseases examined by indirect linkage analysis

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ABSTRACT

BACKGROUND: Many centers of assisted reproduction in the Czech Republic offer preimplantation genetic diagnosis with fluorescent in situ hybridization (FISH) to couples requiring preimplantation genetic diagnosis (PGD) of X-linked diseases. However, this process results in discarding all male embryos and is not able to distinguish a carrier or healthy female embryo in X-linked recessive disorders.

OBJECTIVES: The main aim of this study was to summarize a six-year period of PGD of X-linked monogenic diseases using indirect linkage analysis.

METHODS AND RESULTS: We wanted to accentuate the advantage indirect analysis of PGD using multiple displacement amplification (MDA) followed by short tandem repeat (STR) analysis. We present forty-six PGD cycles, including pre-case haplotyping (PGH) panel, for fifteen X-linked diseases. Embryo transfer was made thirty-eight times and gravidity was confirmed in thirteen female probands with a success rate of pregnancy calculated at 42 %.

CONCLUSIONS**:** PGD procedure using MDA amplification followed by STR analysis provides help in identifying genetic defects within embryos prior to implantation. The reliability of the method was also supported by high pregnancy rate compared to other publications, which commonly achieved a 30–35 % success rate *(Tab. 2, Fig. 1, Ref. 33)*. Text in PDF *www.elis.sk.*

KEY WORDS: indirect diagnosis**,** X-linked inheritance, single gene mutation, haplotype analysis.

Abbreviations: ADO – Allelic Dropout, DOP-PCR – Degenerate Oligonucleotide PCR, FBH – Fetal Heart Heat, FISH – Fluorescent In Situ Hybridisation, IVF – In Vitro Fertilisation, MALBAC – Multiple Annealing and Looping Based Amplification Cycles, MDA – Multiple Displacement Amplification, STR – Short Tandem Repeat, PCR – Polymerase Chain Reaction, PEP – Primer Extension Pre-amplification, PGD – Preimplantation Genetic Diagnosis, PGH – Pre-case Genetic Haplotyping, WGA – Whole Genome Amplification

Introduction

Monogenic disorders arise as a direct consequence of a single gene being defective. These disorders exhibit an inheritance pattern and were initially classified into three groups; autosomal dominant, autosomal recessive and X-linked. The concepts of dominance and recessiveness were later applied to sex-linked traits, based on the phenotype in heterozygous females (1, 2, 3).

Approximately 1100 genes are thought to be located on the X chromosome, of which approximately 40 % are known to be associated with disease phenotypes (2). Most of them are classified as recessive (4), a much smaller number are classified as dominant (5) and a few are classified as dominant and lethal in hemizygous males (1, 6, 7).

PGD (Preimplantation Genetic Diagnosis) is a possible approach for incoming parents with known heritable X-linked disorders in the family to achieve an uncomplicated pregnancy and the birth of a healthy baby.

Many centers of assisted reproduction in the Czech Republic offer PGD using fluorescent in situ hybridization (FISH) for achieving a healthy pregnancy. However, PGD by FISH only diagnoses embryos of female or male sex and is unable to identify specific genotypes of affected/ healthy male or carrier/ healthy female embryos in recessive disorders and affected/ healthy female embryos in dominant disorders. Although FISH previously had superseded PCR for sex determination, currently, the specific diagnosis of single gene defects remains dependent largely on DNA amplification with PCR. In the case of X-linked disorders, testing of a specific gene has the advantage of ensuring that all embryos free of the mutated gene can be recommended for implantation, irrespective of sex assessment (8, 9, 10).

In our centre, we provide PGD for all monogenic diseases depending on the genetic burden of the reproductive couple. We have performed over 260 PGD cycles for various genetic disorders. From this number, approximately one fifth belonged to X-linked diseases. In this study, we focused on X-linked disorders due to their specific mode of inheritance and because they form the largest group of diseases located on the X chromosome from our list of all examined monogenic disorders. Further, we first performed sequencing analysis of a single cell during PGD

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cycle of X-linked disease to indentify affected or healthy male embryo because of crossing over occurring in the area of the *MTM1* gene.

Materials and methods

Pre-case haplotyping analysis

Before commencing the PGD procedure, the reproductive couples requiring PGD underwent consultation with a clinician, explaining the possible risks of PGD. To initiate PGD analysis for X-linked monogenic disorders, it is important to establish the mutation in the DNA by obtaining biologic material of the immediate family member(s) carrying the mutated haplotype.

All examined couples and their relatives provided written informed consent for participating in PGH pre-case genetic haplotyping (PGH) analysis to identify high-risk (linked to a single gene mutation) and low-risk (without a mutation) haplotype. In the case of children, informed consent was provided by their parents or legal guardians.

Genomic DNA from peripheral blood was extracted using standard protocol. Short tandem repeat (STR) markers for PGH were chosen by their proximity of within 5 centimorgan (cM) in the flanking regions of the causal mutation within the examined gene. The PGH protocol for every family was unique based on informativeness of particular STR markers.

Polymerase chain reaction (PCR) assays using fluorescently labelled primers were performed as two sets (A/B) for each PGH panel of X-linked disease. Each set, containing on average eight polymorphic di/tri/tetra-nucleotide repeat markers, was optimised for specific PCR conditions. Universal STR markers of AMELX/Y, SRY, X22 and DXYS154 (DXYS1107) were inserted in sets for confirmation of female and male sex.

IVF protocols

PGD involves in vitro fertilization (IVF) with control of the processes of oocyte maturation, fertilization and implantation, to select and transfer back to the uterus only healthy embryos (11).

The single biopsied cells (blastomeres) from particular embryos were washed in special solution, lysed and then used directly for multiple displacement amplification (MDA) as described by Renwick et al (12). The main role of MDA (Repli-g kit, Qiagen CZ) is to provide large quantities of DNA from a small amount of material but with a certain risk of allelic dropout (ADO) (13, 14). Allelic dropout was calculated within embryo screening as the number of unamplified alleles from the total number of expected full informative alleles at the heterozygous loci. STR markers (STR2, STR25, STR4, STR44, STR45, STR49, STR62, STR79GT2, STR79GT3, STRMP, 5´DYSII, DXS992, DXS1214, DMDSTR7, DMD3+33, AMXY a SRY) included in the PGH protocol of Duchenne muscular dystrophy (DMD) were used to evaluate the ADO rate.

MDA products were subsequently subjected to PCR assay using fluorescent STR markers to identify embryos with high- and low-risk haplotypes according to PGH panel.

Because of possible contamination and ADO, we carried out each PGD protocol with three negative controls: two MDA blanks (solution control from washing drop, negative control with sterile water) and one blank for every PCR set. As a positive control, samples of partner's DNA isolated from peripheral blood were used.

Blastomeres were biopsied from cleavage-stage embryos on day 3. In the case of ambiguous results of haplotype comparison between parents and embryos by STR analysis (caused by high ADO, low informative STR markers or no cell amplification), the entire process was repeated with trophoectoderm on day 4 for the embryo to be transferred on day 5.

Couple requiring the last PGD cycle of all X-linked disorders had the genetic burden of X-linked myotubular myopathy, where the female proband was a heterozygote of the c.82delA mutation. For preparation of the PGH panel, we involved DNA from both partners and their two male descendants diagnosed with a hemizygous status of the c.82delA mutation. During the PGD cycle, sequencing analysis of a single cell was first carried out for the c.82delA mutation detection. The MDA product was subjected simultaneously to haplotype comparison and *MTM1* gene sequencing analysis.

Tab. 1. The list of the examined X-linked diseases arranged according to the location on chromosome X.

Name of X-linked disease	Gene	Location	Number of PGD cycles	Number of partners
Duchenne muscular dystrophy (DMD)	*dystrophin*	Xp21.1	12	7
Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX)	*FOXP3*	Xp11.23	3	1
X-linked severe combined immunodeficiency (X-SCID)	*IL2RG*	Xq13.1	1	1
X-linked dominant Charcot-Marie-Tooth neuropathy type 1 (CMTX1)	*GJB1,Cx32*	Xq13.1	1	1
Fabry disease	*GLA*	Xq22.1	1	1
Pelizaeus–Merzbacher disease (PMD)	*PLP1*	Xq22.2	0	1
Alport syndrome	*COL4A5*	Xq22.3	5	3
Oculocerebrorenal syndrome of Lowe (OCRL)	*OCRL*	Xq26.1	1	1
Börjeson-Forssman-Lehman Syndrome (BFLS)	*PHF6*	Xq26.2	2	1
Hemophilia B	*Factor IX*	Xq27.1	2	1
Fragile X syndrome (FXS)	*FMR1*	Xq27.3	9	5
Hemophilia A	*Factor VIII*	Xq28	6	4
Incontinentia pigmenti	*NEMO, IKBKG*	Xq28	1	1
X-linked adrenoleukodystrophy (X-ALD)	*ABCD1*	Xq28	1	1
X-linked myotubular myopathy (XLMTM)	*MTM1*	Xq28	1	1

Results

We compiled the results for six years and found fifteen X-linked disorders. From these, twelve were recessively and three dominantly (Incontinentia pigmenti, Fragile X syndrome and the X-linked dominant Charcot-Marie-Tooth neuropathy type 1) inherited diseases. The X-linked disorders are shown in Table 1.

The total number of PGD cycles of X-linked diseases was forty-six. Of the thirty couples who underwent PGD cycle, 23 % were diagnosed with Duchene muscular dystrophy, 17 % were diagnosed with Fragile X syndrome, 13 % were diagnosed with Hemophilia type A and 10 % were diagnosed with Alport syndrome. Eleven couples were diagnosed with the remaining eleven examined X-linked diseases. One couple with PMD disease did not undergo IVF protocol, but only preliminary analysis to identify high-risk haplotype.

DNA amplification from a single cell was performed successfully by MDA. We analyzed two hundred and twenty-five single human blastomeres and only used trophoectoderm for confirmation of the result in ten cases. No amplification was detected in eight blastomeres and two embryos had insufficient informative amplified STR markers to arrive at an unambiguous result. Based on the PCR outcome obtained by using MDA amplification and STR analysis, the ADO rate was calculated as 16 %.

MDA products from blastomeres of single embryos subjected to STR analysis were compared with extracted DNA from both partners. Approximately three quarters of analyzed embryos (160) were determined to be healthy based on carrying low-risk haplotype and high-risk haplotype heterozygosity, valid only for females in X-linked recessive diseases. In X-linked dominant diseases, embryos with high-risk haplotypes were excluded. Other examined embryos (65) were not recommended for transfer due to abnormal results. The results of the analyzed embryos are summarized in Table 2.

Crossing-over (C-O) of chromosome X was detected in twenty-nine cells. The location of the C-O point cannot be accurately detected using STR analysis, but the C-O of two STR markers´ C-O was determined. Hereto, the examined gene localized between informative STR markers; the result depended on the existence of proven high- or low-risk haplotypes within the examined gene. Cases with an identified high-risk haplotype or C-O occurring inside the gene were concluded as affected in male embryos and heterozygous female embryos in X-linked recessive diseases; embryos with proven low-risk haplotypes were recommended for transfer. C-O localized behind/ in front of a gene did not influence the total result.

Tab. 2. The results of the 225 analyzed embryos.

Normal results	Number of embryos
XY-healthy	53
XX-healthy	48
XX-carrier	38
XX-healthy or carrier (C-O inside of the gene)	4
XX-healthy (C-O behind or in front of the gene)	5
XX-carrier (C-O behind or in front of the gene)	7
XY-healthy (C-O behind or in front of the gene)	5
Abnormal results	**Number of embryos**
XX-affected	5
XY-affected	31
XY-affected (C-O inside of the gene)	5
Monosomy X	16
Monosomy X with C-O	3
XXY	4
UPD from mother	1

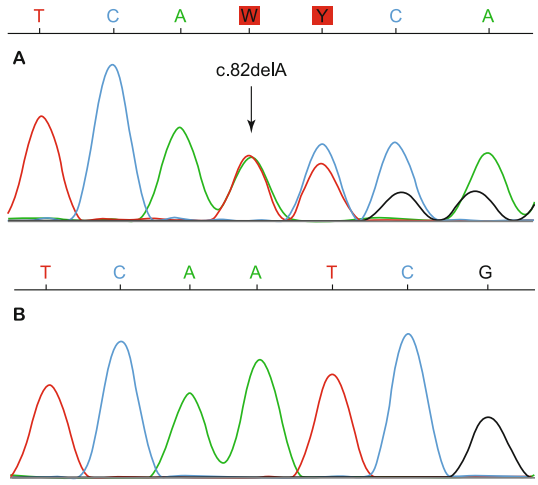

Fig. 1. Sequencing data detection of the c.82delA mutation in the *MTM1* gene. A – Electropherogram with heterozygous result of the c.82delA mutation from female proband blood sample. B – Electropherogram of single cell from three-day male embryo where no mutation was shown.

Numeric aberration of sex chromosomes was determined in twenty-three embryos. Monosomy X was established in nineteen cases, whereas monosomy X with C-O was detected in three blastomeres. Only four embryos were assessed with maternal low-risk haplotype. Paternal low-risk haplotype was identified in three embryos, but they were excluded due to an unknown status of maternal haplotype. Maternal high-risk haplotype of monosomy X was shown in nine affected embryos.

Aneuploidy of chromosomes X and Y was shown in four embryos with genotype XXY.

Uniparental Disomy (UPD) was determined when two maternal X chromosomes (maternal heterodisomy) were shown within STR analysis in a single cell; the embryo was subsequently not recommended for transfer.

In the last PGD cycle of X-linked myotubular myopathy, sequencing analysis was used to identify the X-linked haplotype of the embryo with C-O arising inside of the *MTM1* gene. In our first attempt at sequencing analysis of a single cell, a male proband requiring PGD had *de novo* mutation in the neurofibromatosis type 1 (NF1) gene. We sequenced sperm cells for PGH separately followed by blastomeres within the PGD cycle. The same process used in that study was employed for X-linked disorder. The PGH panel was provided in the standard manner using blood samples of the couple and their immediate family members. As a result, the female proband had only one embryo for examination during the IVF cycle. A male sex with C-O on chromosome X was detected via

STR analysis, but the location of the C-O point did not accurately determine whether it was inside or outside of the *MTM1* gene. Sequencing analysis of the *MTM1* gene was performed with low-risk haplotype (Fig. 1), identifying the embryo as an XY-healthy.

In total, we performed forty-six PGD cycles of X-linked disorders, and embryo transfer was carried out in thirty-eight cases (two embryos were transferred in sixteen IVF cycles and one embryo was transferred in twenty-two IVF cycles). The twenty-five healthy embryos were frozen for the next possible incoming pregnancy of couples with X-linked genetic affliction. Eighty-one embryos were excluded from transfer due to abnormal embryo development determined by an insufficient amount of cells, growth evenness or by fragmentation degree.

We showed a 52 % total pregnancy rate per embryo transfer. The total pregnancy rate included all positive pregnancies from PGD cycles wherein the thirteen female probands had a confirmed pregnancy by fetal heart beat (FBH) and four pregnanciess were only biochemical. After excluding biochemical pregnancy, there was a 42 % successful pregnancy rate as confirmed by FBH. In three cases, FBH revealed twin pregnancy. Four pregnant women spontaneously miscarried within the first trimester, two of which was a twin pregnancy. Independent of IVF cycles, three unprompted pregnancies were confirmed.

Discussion

The PCR-based method and FISH analysis were the most commonly used techniques in PGD (15). However, recently, many new methods for single cell analysis appeared (e.g., comparative genome hybridization – CGH, microarray, karyomapping, new generation sequencing – NGS) and often require specific criteria for DNA quantities and quality. Therefore, whole genome amplification (WGA) techniques are usually necessary to analyze samples with limited quantity, such as a single blastomere.

WGA techniques can be basically divided in two groups: PCR-based methods and isothermal amplifications. Although PCR methods have many advantages, they also have limitations. Nested-PCR, primer extension pre-amplification (PEP) and degenerate oligonucleotide PCR (DOP-PCR) were developed to reduce the disadvantages of conventional PCR for single cell analysis (16, 17). However, the products from these techniques are not available for whole genome research studies and DNA yields are limited (18) compared to isothermal MDA and multiple annealing and looping based amplification cycles (MALBAC).

In addition, PCR-based methods produce incomplete genome coverage of loci due to preferential binding of the primers to specific loci (19, 20, 21). Similarly, these techniques, as well as nested-PCR using the low fidelity enzyme *Taq* polymerase that generates resulting DNA with a much higher mutation rate and can lead to error-prone amplification with a reported error rate of 1 in 9 000 (22, 23).

Isothermal MDA amplification employs hexamer primers binding to the DNA template randomly and, with the help of Φ29 polymerase, the entire nuclear genome is synthetized. Φ29 polymerase has high fidelity and 3'–5' proofreading activity reducing the amplification error rate to 1 in 10^6–10^7 bases (24, 25).

MDA, as well as other WGA methods, have the disadvantage of allelic dropout, which can lead to misdiagnosis of embryo status. We carried out re-analysis of trophoectoderm in 4 % of the examined single blastomeres. Eight cells did not embody amplification, and it may have been caused by DNA deterioration, damage or absence of nuclear DNA in blastomeres and chromosomal nullisomy. In two cases, we performed a second control because only a few informative STR markers had been amplified in blastomeres. We attributed this problem to an obviously high allelic dropout. Additionally, we cannot determine whether ADO of paternal haplotype in STR markers occurred in the four blastomeres where monosomy X with healthy maternal haplotype was determined. Hence, this embryo status of monosomy X was mentioned in the final results of the report as a suggestion for clinical genetics counselling couples.

Based on the outcomes of MDA amplification with subsequent PCR assay, we recorded a 16% ADO rate in accordance with other studies (26-29), presenting a 5-31 % range of ADO rate using the same PGD procedure.

Sermon and de Rycke (16) suggested possible ADO occurrence depending on the cell type analyzed, cell lysis specifications and the PCR conditions. Some reports (11, 15, 30) have presented that blastomeres exhibit a greater ADO rate with lower amplification bias than do other cell types (lymphocytes, polar body and fibroblast), but such a difference has not been unanimously confirmed, as in the Glentis et al (31) study. Fortunately, several other innovations to improve the accuracy and efficiency of single cell analysis have emerged for cell lysis specification and PCR conditions.

Kim et al (32) compared five different lysis buffers for decreasing ADO and single cell analysis using an alkaline lysis buffer resulting in more efficient amplification bias and a lower ADO rate than the other methods tested. In addition to reducing ADO, Thornhill and Snow (15) and Verlinsky and Kuliev (11) recommended using one or two polymorphically linked markers in PCR assay reducing undetected ADO by approximately 50 and 75 %, respectively. With three linked markers, ADO can be virtually always detected. The use of more polymorphically linked markers in one PGH panel can also potentially increase the number of embryos available for transfer, especially in X-linked recessive disorders (18).

Our PGD procedure for X-linked monogenic diseases was drawn from the Renwick et al (12) study that first revealed the advantage of PGD using indirect linkage analysis, as well as a description of ADO minimalisation complying with recommended references mentioned above: blastomere lysis using buffer with NaOH and PCR assay containing sixteen polymorphically linked STR markers per PGH panel. Another benefit of indirect linkage analysis is that designed STR markers from one PGH panel of X-linked monogenic disease can be used for all families even when there are a variety of pathogenic mutations in a single gene.

Although the PGD procedure can be stringently optimised, we have modified the strategy of PGD procedure to shorten the MDA reaction time from 16 to 4 and then to 2 hours. It was shown to be highly efficient for ADO reduction and, moreover, using a blastomere taken from the cleavage-stage embryo, reporting of the final result of the embryo status is accomplished within the same day. Both Lau et al (33) and we modified MDA reaction to 4 hours and ob-

tained a 10 % ADO rate. This modified strategy appears hopeful for improving efficiency for PGD procedures of monogenic diseases.

Conclusion

PGD procedure using MDA amplification followed by STR analysis provides help in identifying genetic defects within embryos prior to implantation. The reliability of the method was also supported by a high 42 % pregnancy rate compared to other publications, which commonly achieved a 30–35 % success rate. Each PGD procedure requires optimization and use of new, modified strategies can help achieve high efficiency and accuracy in many other diagnostic approaches.

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