Journal of Assisted Reproduction and Genetics (2021) 38:2893–2908
https://doi.org/10.1007/s10815-021-02312-z

GENETICS

# *NLRP7* variants in spontaneous abortions with multilocus imprinting disturbances from women with recurrent pregnancy loss

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Received: 19 April 2021 / Accepted: 2 September 2021 / Published online: 23 September 2021


## Abstract

**Purpose** Comparative analysis of multilocus imprinting disturbances (MLIDs) in miscarriages from women with sporadic (SPL) and recurrent pregnancy loss (RPL) and identification of variants in the imprinting control gene *NLRP7* that may lead to MLIDs.

**Methods** Chorionic cytotrophoblast and extraembryonic mesoderm samples from first-trimester miscarriages were evaluated in 120 women with RPL and 134 women with SPL; 100 induced abortions were analyzed as a control group. All miscarriages had a normal karyotype. Epimutations in 7 imprinted genes were detected using methyl-specific PCR and confirmed with DNA pyrosequencing. Sequencing of all 13 exons and adjusted intron regions of the *NLRP7* gene was performed.

**Results** Epimutations in imprinted genes were more frequently detected ($p < 0.01$) in the placental tissues of miscarriages from women with RPL (7.1%) than in those of women with SPL (2.7%). The predominant epimutation was postzygotic hypomethylation of maternal alleles of imprinted genes (RPL, 5.0%; SPL, 2.1%; $p < 0.01$). The frequency of MLID was higher among miscarriages from women with RPL than among miscarriages from women with SPL (1.7% and 0.4%, respectively, $p < 0.01$). Variants in *NLRP7* were detected only in miscarriages from women with RPL. An analysis of the parental origin of *NLRP7* variants revealed heterozygous carriers in families with RPL who exhibited spontaneous abortions with MLIDs and compound heterozygosity for *NLRP7* variants.

**Conclusion** RPL is associated with *NLRP7* variants that lead to germinal and postzygotic MLIDs that are incompatible with normal embryo development.

**Trial registration:** Not applicable.

**Keywords** Recurrent pregnancy loss · Placenta · DNA methylation · Trophoblast · *NLRP7* · Multilocus imprinting disturbances (MLID)

## Introduction

Pregnancy loss is one of the most important problems in reproductive medicine. It has been established that 15–20% of all clinically diagnosed pregnancies end in spontaneous abortion, 75–80% of which show a gestational age of 12 weeks or less. Two or more subsequent miscarriages with a gestational age up to 22 weeks constitute recurrent pregnancy loss (RPL) according to the WHO definition and the American Society for Reproductive Medicine (ASRM), which occurs in 2–5% of all married couples [1].

Genetic factors play the most significant role in disrupting early embryonic development. Cytogenetic abnormalities are detected in 50% of abortions on average [2]. The factors contributing to such a high mortality rate can also include neuroendocrine disorders and maternal infectious diseases, age, immune conflicts between the mother and fetus, and adverse environmental effects. However, the reasons for pregnancy loss excluding the abovementioned factors are unclear in 40% of women.

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The presence of a normal karyotype in an embryo does not necessarily imply a balanced genome because of genomic imprinting. Imprinting determines the differential monoallelic expression of genes depending on their parental origin; it is inherited over generations and results in the functional noncomplementarity of the maternal and paternal genomes. The differential expression of parental genes is determined by the DNA methylation and covalent modification of chromatin histone's proteins. Approximately 100 imprinted loci have been found in the human genome to date; these loci are involved in the feto-placental growth of mammals by controlling cell proliferation and differentiation, hormone metabolism, and the regulation of growth factors . Thus, they are essential for normal human embryonic development [3].

One of the mechanisms that is generally considered to disrupt the dose of imprinted genes in the course of early embryonic development is the uniparental disomy (UPD) of whole chromosomes (erroneous inheritance of both homologous chromosomes from one parent). UPD leads to functional nullisomy or disomy of critical imprinted genes for normal development. However, a series of studies searching for UPD in spontaneous abortions have not shown a significant contribution of this phenomenon to the etiology of early embryonic developmental arrest [4–6]. Taking into account the epigenetic nature of imprinting, it can be hypothesized that the expected negative effects of imprinted gene disorders in early human embryogenesis would be associated with aberrant epigenetic chromatin modifications that do not change the nucleotide sequence of the gene itself (epimutations) rather than with chromosome segregation errors leading to UPD. For the affected gene, the epimutation effects would of course be functionally equivalent to the UPD of chromosomes containing imprinted genes. Thus, if the imprinted allele of an inactive parental homolog is hypomethylated, it will be activated, and biallelic expression will occur. In contrast, epimutations associated with the hypermethylation of an active allele will lead to a complete absence of the products of the imprinted gene in a cell.

There are currently four different types of molecular alterations that are known to potentially cause imprinting disturbances: (i) gene variants that inactivate a single expressed allele; (ii) copy number variations (CNVs) involving imprinted genes (generally microdeletions or microduplications of chromosome regions containing imprinted genes); (iii) complete or segmental UPD of chromosomes; and (iv) epimutations, commonly comprising alterations of 5mC methylation, including either the loss or gain of methylation. These genetic variants are recorded during both prenatal and postnatal human development. However, in the postnatal period, CNVs predominate (53%), while in the prenatal period, there is selection against epimutations in imprinted genes (65%); selection acts primarily against regulatory and potentially reversible epigenetic modifications and not against structural abnormalities such as deletions, UPD, or gene variants [7].

There are currently available data on epimutations of imprinting centers and imprinted genes in human spontaneous abortions [8–10], intrauterine growth restriction [11, 12], and stillbirth cases after both assisted and natural conception [13, 14]. The analysis of these data showed that epimutations are one of the potential mechanisms causing genomic imprinting abnormalities that lead to pathologies of embryonic development. Additionally, they arise with a relatively high frequency, being found in 4–18% of miscarriages. Finally, some spontaneous abortions show multilocus imprinting disturbances (MLIDs). At the same time, MLIDs are not restricted solely by prenatal development. Multiple epimutations have also been described in patients with Beckwith–Wiedemann syndrome (BWS), Silver–Russell syndrome (SRS), pseudoparathyroidism 1B, Temple syndrome, and transient neonatal diabetes mellitus [15].

The presence of MLID suggests the existence of variants in genes that control imprinting in humans. This hypothesis is supported by data on the association of epimutations in imprinted genes with the occurrence of variants in various genes, such as *NLRP7* (*NALP7*), *KHDC3L*, *NLRP5*, *NLRP2*, *ZFP57*, and *PADI6* [16]. Among these genes, variants in *NLRP7*, *KHDC3L*, *NLRP5*, *NLRP2*, and *PADI6* are accompanied by MLIDs and fetal development arrest. *NLRP7* and *KHDC3L* variants have been found in familial and recurrent biparental complete hydatidiform moles (BiHMs) accompanied by a global disruption of methylation establishment among imprinted genes during oogenesis [17]. In addition, the *NLRP7*, *KHDC3L*, *NLRP5*, *NLRP2*, and *PADI6* gene products are included in the subcortical maternal complex (SCMC), which is a multiprotein complex that is uniquely expressed in mammalian oocytes and early embryos and is essential for zygote progression beyond the first embryonic cell divisions [18].

In the context of RPL (not accompanied by hydatidiform mole), no data on the prevalence of *NLRP7* variants have been reported to date. Therefore, the aim of this study was to compare MLIDs in miscarriages from women showing sporadic pregnancy loss (SPL) or RPL and to search for genetic variants in the imprinting control gene *NLRP7* that may lead to MLID in miscarriages.

## Materials and methods

### Clinical samples

The study was performed using placental tissues (chorionic cytotrophoblast and extraembryonic mesoderm) from miscarriages in the first trimester of pregnancy obtained from

women showing RPL (RPL group, 120 miscarriages) or sporadic miscarriage (SPL) (loss of only one pregnancy in anamnesis; SPL group, 134 miscarriages). One hundred induced abortions were analyzed as a control group. Informed consent for participation in this study was received from all couples. All the patients were Russians. The gestational age of miscarriages in the compared groups was not significantly different (Table 1). The mean maternal and paternal ages also did not differ between the groups (Table 1). This study was approved by the local ethics committee (Protocol # 6 from 08.11.2012).

## Genetic studies

The karyotypes of the miscarriages were established using conventional metaphase analysis or a combination of comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) methods, as described previously [19, 20]. All miscarriages selected for the study had a normal karyotype. The methylation status of CpG dinucleotides was determined in genomic DNA extracted from chorionic cytotrophoblasts and extraembryonic mesoderm specimens. To increase the number of chorionic cytotrophoblast cells, we performed maceration of chorionic villi that were obtained after mechanical separation of the extraembryonic tissues under an Axiovert 200 inverted microscope (Carl Zeiss, Germany) and treatment with 70% acetic acid for 3–5 min followed by three washes of the obtained cell suspension with PBS according to a modified protocol [21]. DNA was isolated using a standard phenol–chloroform extraction. This method allows the isolation of up to 900 ng of chorionic cytotrophoblast DNA. Genomic DNA was also extracted from the peripheral blood from mothers and fathers using the standard phenol–chloroform method.

Epimutations in the imprinted differentially methylated regions (DMRs) of the *PEG1/MEST*, *PEG3*, *PEG10*, *DLK1*, *PLAGL1*, *KCNQ1OT1*, and *GRB10* imprinted genes were detected with methyl-specific PCR and DNA pyrosequencing (Pyromark Q24, QIAGEN, Germany) using the primers listed in Table S1. The number and position of each CpG in the DMR for each imprinted gene are presented in Table S2. For methylation analyses, bisulfite conversion of DNA was performed using the EZ DNA Methylation Direct Kit (Zymo Research, USA) according to the manufacturer's protocol. The PCR samples contained a normal stabilizing buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μL forward and reverse primers (10 pmol each), 2 units of HotStart Taq polymerase (Qiagen), and 1 μL DNA (after bisulfite conversion) in a total volume of 25 μL. For pyrosequencing, the biotinylated PCR product was subsequently purified, and the single strand DNA was immobilized according to the manufacturer's recommendations. Next, 0.3 μmol of the pyrosequencing primer was added to each sample, and the reaction was run using the PyroMark Q24 pyrosequencer (Qiagen). The methylation index was calculated as the ratio of methylated cytosine and the sum of methylated and non-methylated cytosines using PyroMark Q24 software at the CpG sites of the promoter regions of the imprinted genes.

Using the methylation array GoldenGate Methylation Cancer Panel I (Illumina, USA), we previously showed that epimutations in the imprinted genes *DLK1* (14q32.2), *PEG10* (7q21.3), *PLAGL1* (6q24.2), *KCNQ1OT1 (KvDMR)* (11p15.5), *PEG3* (19q13.43), *PEG1/MEST* (7q32.2), and *GRB10* (7p12.1) selected for this study were associated with spontaneous abortion in the first trimester of pregnancy [22]. The genes *DLK1*, *PEG10*, *PLAGL1*, *KCNQ1OT1*, *PEG3*, and *PEG1/MEST* are expressed only by the paternal allele, in contrast to the *GRB10* gene, which is expressed in extraembryonic tissues by the maternal homolog. Therefore, hypomethylation of the maternal allele and hypermethylation of the paternal homolog can affect 6 of these genes. Similarly, hypermethylation of the maternal allele and hypomethylation of the paternal homolog can be associated with epimutations of a single *GRB10* gene.

The criterion for the selection of tissue (chorionic cytotrophoblast and extraembryonic mesoderm) for this study was that the cytotrophoblast of the chorionic villi (the stem cell line of all future placental cells that gives rise to an invasive trophoblast and syncytiotrophoblast) is derived from the trophectoderm, i.e., the external blastocyst layer. However, the stroma of the chorionic villi is layered with extraembryonic mesoderm cells derived from the epiblast, which in turn descends from the inner cell mass and later gives rise to all embryonic tissues. Therefore, the methylation index of the DMRs of imprinted genes in spontaneous abortions from women with sporadic miscarriages and RPL was analyzed for chorionic cytotrophoblast and extraembryonic mesoderm. The comparative analysis of the epimutation distribution in the cytotrophoblast and extraembryonic mesoderm tissues made it possible to identify somatic (present in only one tissue) and germinal (present in both tissues) abnormalities in the methylation pattern.

Considering the specificity of epigenetic reprogramming, in the case of the hypermethylation or hypomethylation of imprinted genes in only one of the examined

**Table 1** Characteristics of the studied groups

| Group parameter | RPL | SPL | Control |
|---|---|---|---|
| Gestational age of miscarriages (weeks) | 7.9 ± 1.12 | 8.1 ± 1.21 | 7.6 ± 0.92 |
| Mean maternal age (years) | 30.2 ± 3.41 | 29.7 ± 5.24 | 26.4 ± 2.03 |
| Mean paternal age (years) | 36.5 ± 4.21 | 33.6 ± 4.12 | 30.8 ± 6.14 |

Note: There was no significant difference among the three groups in the gestational age of miscarriages or the mean maternal and paternal ages ($p > 0.05$)

tissues (cytotrophoblast or extraembryonic mesoderm), the epimutations were considered only to have a postimplantation origin, resulting from disturbances in the maintenance of imprinting mechanisms in embryonic somatic cells. In contrast, the presence of epimutations in both tissues was considered to be due to reprogramming disturbances in the primordial parental germ cells. Therefore, such epimutations were considered germinal. In the case of hypomethylation at the examined locus in both tissues, it was impossible to unequivocally determine the origin of the epimutation, as such a methylation pattern could be established either in the gametes of the parents or owing to the loss of stability at differentially methylated sites for epigenetic genome reprogramming during cleavage divisions.

In reporting the methylation index and epimutations of the imprinted genes, we applied recommendations for imprinted DMRs according to the Nomenclature group of the European Network for Human Congenital Imprinting Disorders [23]. Information regarding the methylation index of the imprinted DMRs and all CpG positions in the genomes of the control group and in miscarriages from women with SPL and RPL is presented in Table S2.

The spectrum of epimutations was registered according to the following parameters and combinations thereof:

- hypo- or hypermethylation of alleles;
- presence of epimutations on chromosomes of maternal or paternal origin;
- germinal or somatic origin of epimutations.

The epimutation frequency was determined based on the total numbers of the examined miscarriages (134 and 120 abortions in the SPL and RPL groups, respectively), imprinted genes (7), and 40 CpGs (ranging from 5 CpGs in *DLK1*, *PLAGL1*, *PEG1/MEST* and *PEG3*; to 6 CpGs in *PEG10* and *KCNQ1OT1*; and to 8 CpGs in *GRB10*) in both groups; the analysis of two tissues from each miscarriage; and the probability of epimutations at each locus on chromosomes of maternal and paternal origin. Therefore, the total number of examined CpG dinucleotides was 9600 ($40\times120\times2$) in the RPL group and 10720 ($40\times134\times2$) in the SPL group. The MLID frequency was determined based on the total numbers of examined miscarriages (134 and 120 abortions in the SPL and RPL groups, respectively), imprinted genes (7), and the analysis of two tissues from each miscarriage. Therefore, the total number of examined samples was 1680 ($120\times7\times2$) in the RPL group and 1876 ($134\times7\times2$) in the SPL group.

All 13 exons and the adjusted intron regions of the *NLRP7* gene were sequenced in 29 spontaneous abortions with MLIDs from women with RPL and 7 spontaneous abortions from women with sporadic miscarriage. We developed a primer panel for the analysis of the complete sequence of the *NLRP7* gene, transcript number: ENST00000588756.5 (Table S3). The DNA samples were amplified via long-range PCR using the BioMaster LR HS-PCR-Color (2×) kit (Biolabmix, Russia) on a SureCycler 8800 thermocycler (Agilent Technologies, USA). Amplification was conducted according to the following program: 94 °C for 4 min; 10 cycles of 94 °C for 20 s, 57 °C for 30 s, and 68 °C for 13 min; and 20 cycles of 94 °C for 20 s, 57 °C for 30 s, and 68 °C for 13 min (+ 10 s per cycle). The presence of amplified products was verified using agarose gel electrophoresis.

DNA libraries were prepared using the Nextera Flex kit (Illumina, USA) according to the protocol recommended by the manufacturer. Gene sequencing was carried out on a MiSeq sequencer (Illumina, USA) according to the protocol recommended by the manufacturer. The data obtained after sequencing were subjected to bioinformatics processing, which included the following key steps: evaluation of read quality using the FastQC program, removal of adapters and trimming of the sequence with the Trimmomatic-0.36 software package, alignment to target sequences using Bowtie2, conversion to BAM files, and sorting and conversion to VCF format using a set of SAMtools utilities. The pathogenicity of the identified variants was predicted using online tools according to the recommendations of the genetic variant nomenclature following Human Genome Variation Society conventions and the American College of Medical Genetics and Genomics (ACMG) for the interpretation of NGS [24, 25]. The identified genetic variants were confirmed by Sanger sequencing (ABI 3130XL, Applied Biosystems, USA) using the primers indicated in Table S4. To visualize the detected *NLRP7* variants, we performed 3D modeling of proteins using the SWISS-MODEL Repository (https://swissmodel.expasy.org/).

To exclude population polymorphisms, an analysis was performed on the distribution of the identified *NLRP7* variants from our whole genome data on 700 population samples assessed using HiSeq 2500 (Illumina, USA) (data are available upon request).

Statistical analysis of the data was performed using the $\chi^2$ test and Fisher's exact test.

All molecular genetic studies were performed at the Core Facility 'Medical Genomics' of the Tomsk National Research Medical Center of the Russian Academy of Sciences using the resources of the biocollection 'Biobank of the Population of Northern Eurasia'.

## Results

At the beginning of our study, the methylation status of imprinted genes in the induced abortions (control group) was investigated (Table 2). This analysis showed that the methylation index of the DMRs of all analyzed imprinted

**Table 2** Methylation index of DMRs of imprinted genes in the control group

| Genes | Imprinted DMR name | CpGs | Methylation index (%) | | |
|---|---|---|---|---|---|
| | | | Minimum | Maximum | Average |
| *PEG1/MEST* | *MEST*:alt-TSS-DMR | 5 | 40.1 | 59.2 | 52.24 ± 3.03 |
| *PEG3* | *PEG3*:TSS-DMR | 6 | 41.1 | 66.6 | 53.9 ± 2.76 |
| *GRB10* | *GRB10*:alt-TSS-DMR | 8 | 44.1 | 67.3 | 53.21 ± 2.43 |
| *PLAGL1* | *PLAGL1*:alt-TSS-DMR | 5 | 42.2 | 58.8 | 52.3 ± 2.87 |
| *MEG3/DLK1* | *MEG3/DLK1*:IG-DMR | 5 | 41.1 | 58.9 | 51.5 ± 3.70 |
| *PEG10* | *PEG10*:TSS-DMR | 5 | 40.0 | 68.5 | 53.1 ± 3.65 |
| *KCNQ1OT1* | *KCNQ1OT1*:TSS-DMR | 6 | 46.8 | 69.2 | 55.2 ± 2.94 |

genes corresponded well to the expected monoallelic methylation typical for most imprinted genes. In contrast to SPL, the methylation index of the DMRs of imprinted genes in the spontaneous abortions from women with RPL showed a significant decrease in the methylation indices of DMRs for four imprinted genes: *PEG3*:TSS-DMR, *PLAGL1*:alt-TSS-DMR, *MEG3/DLK1*:IG-DMR and *KCNQ1OT1*:TSS-DMR ($p < 0.05$, Table 3). These genes expressed the paternal allele only. A decrease in the methylation index of DMRs in maternally imprinted genes can lead to biparental expression, loss of imprinting and an increase in the amount of protein product from the moternal allele.

Overall, epimutations of imprinted genes in miscarriages were detected more often in the RPL group than in the SPL group. The total frequency of epimutations in the RPL group was 7.1% per locus (681 of 9600 CpGs), whereas that in the SPL group was 2.7% per locus (293 of 10,720 CpGs) ($p < 0.01$) (Table 4).

Germinal and somatic epimutations were present in both analyzed groups. However, they were significantly more frequent in the RPL group than in the SPL group (Table 4). Most epimutations consisted of hypomethylation of inactive paternal or maternal imprinted alleles. Thus, the frequency of hypomethylation among alleles of maternal origin in the RPL group was significantly higher than that in the SPL group (5.0% ($384/32 \times 120 \times 2$) and 2.1% ($180/32 \times 134 \times 2$), respectively, $p < 0.01$) (Table 4). The total frequency of hypomethylation among paternal alleles was also higher in the RPL group than in the SPL group (3.3% ($64/8 \times 120 \times 2$) and 2.2% ($47/8 \times 134 \times 2$), respectively), but the difference was not statistically significant ($p = 0.8$).

Statistically significant differences in the hypermethylation of paternal alleles, which should theoretically lead to a lack of gene expression, were also shown between the studied groups. The frequency of hypermethylation of paternal alleles in the RPL group was 2.2% ($169/32 \times 120 \times 2$) and that in the SPL group was 0.6% ($51/32 \times 134 \times 2$), $p = 0.005$ (Table 4). Therefore, somatic hypomethylation of normally methylated and unexpressed maternal alleles was predominantly associated with the arrest of embryonic development among miscarriages in the RPL group.

Hypomethylation of *KCNQ1OT1*, *PEG1*, *PEG3*, and *GRB10* and hypermethylation of *DLK1* and *PEG1* were observed more frequently in the RPL group than in the SPL group ($p < 0.05$) (Fig. 1). In addition, *KCNQ1OT1* and *PEG10* were characterized by somatic hypomethylation, whereas *DLK1* and PEG1 were characterized by somatic hypermethylation.

In 36 spontaneous abortions from the two analyzed groups, epimutations were detected at several imprinted loci (MLIDs). In the SPL group, 7 of 134 abortions

**Table 3** Methylation index of DMRs of imprinted genes in spontaneously aborted tissue from women with sporadic miscarriage and recurrent pregnancy loss

| Gene | Imprinted DMR name | Methylation index (%) | | |
|---|---|---|---|---|
| | | SPL group | RPL group | P |
| *PEG1/MEST* | *MEST*:alt-TSS-DMR | 54.01 ± 6.04 | 55.3 ± 11.44 | 0.8 |
| *PEG3* | *PEG3*:TSS-DMR | 52.1 ± 6.17 | 47.2 ± 9.43 | 0.04 |
| *GRB10* | *GRB10*:alt-TSS-DMR | 51.1 ± 4.30 | 51.0 ± 9.47 | 0.9 |
| *PLAGL1* | *PLAGL1*:alt-TSS-DMR | 50.0 ± 4.24 | 45.6 ± 5.25 | 0.04 |
| *MEG3/DLK1* | *MEG3/DLK1*:IG-DMR | 51.2 ± 5.55 | 47.2 ± 8.87 | 0.04 |
| *PEG10* | *PEG10*:TSS-DMR | 53.0 ± 4.68 | 51.0 ± 7.50 | 0.7 |
| *KCNQ1OT1* | *KCNQ1OT1*:TSS-DMR | 52.0 ± 5.37 | 46.7 ± 10.42 | 0.04 |

Note: The SPL group includes women with sporadic miscarriage; the RPL group includes women with recurrent pregnancy loss

**Table 4** Frequency of epimutations of imprinted genes in miscarriages from women with recurrent and sporadic pregnancy loss

| Types of epimutation | Number of loci with epimutations (frequency) in miscarriages | | |
|---|---|---|---|
| | RPL group, 120 miscarriages all 9600 CpG dinucleotides | SPL group, 134 miscarriages all 10720 dinucleotides | p |
| **Hypomethylation of maternal alleles** | | | |
| | Number of normally methylated paternal alleles in each miscarriage | | |
| | 6 genes (32 CpG) | | |
| Somatic | 292 (3.8%) | 154 (1.8%) | 0.005 |
| Germinal or somatic | 92 (1.2%) | 26 (0.3%) | 0.1 |
| Total | 384 (5.0%) | 180 (2.1%) | 0.001 |
| **Hypomethylation of paternal alleles** | | | |
| | Number of normally methylated paternal alleles in each miscarriage | | |
| | 1 gene (8 CpG) | | |
| Germinal | - | - | - |
| Somatic | 64 (3.3%) | 47 (2.2%) | 0.8 |
| Germinal or somatic | - | - | - |
| Total | 64 (3.3%) | 47 (2.2%) | 0.8 |
| **Hypermethylation of maternal alleles** | | | |
| | Number of normally methylated maternal alleles in each miscarriage | | |
| | 1 gene (8 CpG) | | |
| Germinal | 16 (0.8%) | - | 0.9 |
| Somatic | 48 (2.5%) | 15 (0.7%) | 0.1 |
| Total: | 64 (3.3%) | 15 (0.7%) | 0.1 |
| **Hypermethylation of paternal alleles** | | | |
| | Number of normally methylated paternal alleles in each miscarriage | | |
| | 6 genes (32 CpG) | | |
| Germinal | 39 (0.5%) | - | 0.1 |
| Somatic | 130 (1.7%) | 51 (0.6%) | 0.005 |
| Total | 169 (2.2%) | 51 (0.6%) | 0.005 |
| **Frequency of epimutations** | | | |
| | Number of normally methylated paternal and maternal alleles in each miscarriage | | |
| | 7 genes (40 CpG) | | |
| Germinal | 55 (0.5%) | - | 0.001 |
| Somatic | 534 (5.6%) | 267 (2.3%) | 0.001 |
| Germinal or somatic | 92 (1.0%) | 26 (0.3%) | 0.005 |
| Total | 681 (7.1%) | 293 (2.7%) | 0.001 |

Note: *RPL*, recurrent pregnancy loss; *SPL*, sporadic pregnancy loss

simultaneously exhibited MLIDs at two genes. The total frequency of MLIDs in this group was 0.37%. In the RPL group, 29 of the 120 miscarriages showed MLID. The detected epimutations affected 2 to 5 genes simultaneously. The total frequency of MLIDs in this group was 1.72%. (Table 5). Therefore, among the miscarriages from mothers with RPL, MLIDs were recorded significantly ($p = 0.0001$) more often than in the miscarriages from mothers who had experienced only one miscarriage.

MLID indicates the possible existence of special regulatory mechanisms that control the epigenetic status of multiple imprinted loci. In this context, we hypothesized that the presence of some genetic variants in the *NLRP7* gene (19q13.42) in miscarriages may be the cause of MLID, resulting in the disruption of development in the early stages of embryogenesis. Therefore, the *NLRP7* gene was sequenced in the samples from miscarriages showing MLID, which included 29 spontaneous abortions from the RPL group and 7 abortions from the SPL group.

*NLRP7* variants were detected in 7 miscarriages, all from the RPL group. The spectrum and position of the detected genetic variants are presented in Table 6 and Fig. 2. Most of the genetic variants were missense variants in exons 5, 6, 8, and 9, resulting in the substitution of one amino acid for

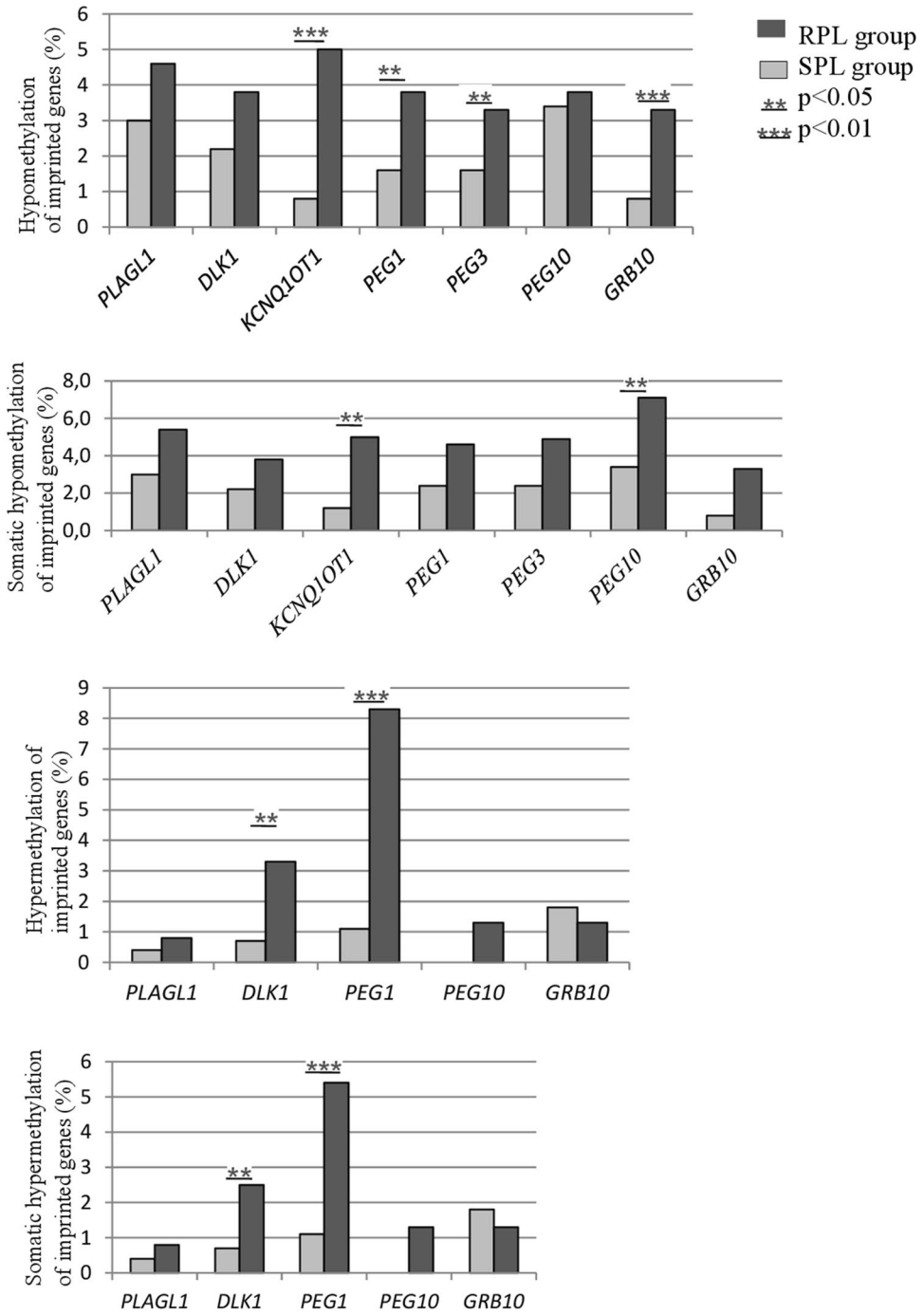


**Fig. 1** Frequency of epimutations in imprinted genes in miscarriages from women with recurrent and sporadic pregnancy loss

another. Three of the detected substitutions (c.2324C>T:p.P775L, rs542783229; c.2426T>C:p.L809P, rs754189651 and c.2396A>G:p.N799S, rs556133069) have been previously described as variants occurring in a normal population. All of these genetic variants were inherited from the mother (miscarriages # 1, 3, and 5). In addition, a single-nucleotide deletion in exons 8 or 10 (spontaneous abortions # 4 and 6) was revealed in two miscarriages (c.2138delA and 2484delT, respectively). A single-nucleotide insertion in exon 10 was also detected in one miscarriage (c.2479insT). Both deletion and insertion genetic variants led to shortening and a change in the NLRP7 protein conformation (Suppl. Figures 1, 2, 3). Three of the detected variants were described for the first time: c.1267C>T:p.L423M, c.655G>A:p.A219T and c.986A>T:p.R329M. All of the miscarriages with *NLRP7* exhibited epimutations in at least three imprinted genes, taking the form of either hypomethylation or hypermethylation (Table 6). Population polymorphisms of detected gene variants were excluded by whole genome data analysis available

**Table 5** Frequency of miscarriages with multilocus imprinting disturbancese (MLIDs)

| Number of affected imprinted genes | Miscarriages in RPL group (120 total miscarriages, two placental tissues) | Miscarriages in SPL group (134 total miscarriages, two placental tissues) |
|---|---|---|
| 2/7 | 15 (0.89%) | 7 (2.6%) |
| 3/7 | 6 (0.35%) | - |
| 4/7 | 6 (0.35%) | - |
| 5/7 | 2 (0.13%) | - |
| Total* | 29 (1.72%) | 7 (0.37%) |

*p = 0.0001

for 700 individuals from the same population (data are available upon request).

For 3 of the 7 miscarriages with *NLRP7* variants, DNA samples were available from both parents.

Miscarriage No 1. The gestational age at the time of spontaneous abortion according to ultrasound examination was 7.5 weeks. This miscarriage showed germinal hypermethylation at *PEG1* and simultaneous somatic hypomethylation at three imprinted genes, *PEG10*, *DLK1*, and *PLAGL1* (Tables 6, 7 and Fig. 3). All of these imprinted genes are expressed only from the paternal allele. In this family, the mother was 29 years old and had a diagnosis of RPL. The father was 32 years old. The mother had a history of three miscarriages at 6–8 weeks of gestation. Cytogenetic analysis of the extraembryonic mesoderm and chorionic cytotrophoblasts showed a normal karyotype (46,XX). The sequencing of *NLRP7* revealed compound heterozygosity of missense variants

**Table 6** Genetic variants in *NLRP7* in miscarriages with multilocus imprinting disturbances

| No M | Reproductive history | MLID | | Exon | Locus reference genomic sequence, protein description | Variant type | Status | Inheritance |
|---|---|---|---|---|---|---|---|---|
| | | Hypermethylated loci | Hypomethylated loci | | | | | |
| 1 | 3 MA | *PEG1* | *PEG10*, *DLK1*, *PLAGL1* | 6 | NM_001127255.1:c.1267C>A:p.L423M | Missense | | Paternal |
| | | | | 9 | NM_001127255.1:c.2324C>T:p.P775L | Missense | rs542783229 | Maternal |
| 2 | 3 MA, 1 AP | *PEG1* | *PEG10*, *KCNQ1OT1* | 5 | NM_001127255.1:c.307C>G:p.P103A | Missense | | N/A |
| | | | | 6 | NM_001127255.1:c.986A>T:p.R329M | Missense | | N/A |
| 3 | 3 MA | *PEG1*, *PEG10* | *KCNQ1OT1*, *PEG3* | 6 | NM_001127255.1:c.655G>A:p.A219T | Missense | | Paternal |
| | | | | 9 | NM_001127255.1:c.2426T>C:p.L809P | Missense | rs754189651 | Maternal |
| 4 | 3 MA | - | *PEG10*, *KCNQ1OT1*, *GRB10* | 8 | NM_001127255.1:c.2138delA | Frameshift | | N/A |
| 5 | 3 MA | *PEG1* | *PEG10*, *DLK1*, *KCNQ1OT1*, *GRB10* | 6 | NM_001127255.1:c.1845A>C:p.Q615H | Missense | | Paternal |
| | | | | 9 | NM_001127255.1:c.2396A>G:p.N799S | Missense | rs556133069 | Maternal |
| 6 | 3 MA, 1 AP | *PEG1* | *DLK1*, *GRB10* | 10 | NM_001127255.1:c.2484delT | Frameshift | | N/A |
| 7 | 3 MA | - | *PEG3*, *DLK1*, *PLAGL1* | 10 | NM_001127255.1:c.2479insT | Frameshift | | N/A |

Note: MA, missed abortions; AP, anembryonic pregnancy; c., DNA coding sequence; Ensembl accession number, http://www.ensembl.org/Homo_sapiens/Transcript/Exons?db=core; g = ENSG00000167634; r = 19:54,923,510–54,941,750; t = ENST00000588756; p., amino acid sequence. The genetic variant nomenclature follows the Human Genome Variation Society and conventions and ACMG [24, 25]

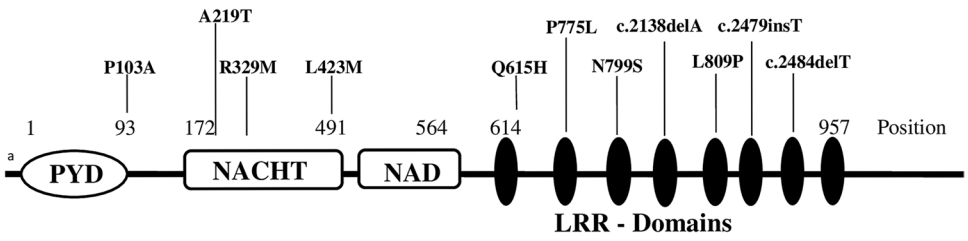


**Fig. 2** The positions of detected genetic variants in *NLRP7*

**Table 7** The origin of epimutations at imprinted loci in spontaneous abortions with variants in the *NLRP7* gene

| Miscarriage No | Variants in *NLRP7* | | Paternally expressed genes | | | | | | | | | | Maternally expressed genes | | Number of genes with epimutations |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *PEG1* | | *PEG10* | *DLK1* | | *PLAGL1* | | *KCNQ1OT1* | | *PEG3* | | *GRB10* | |
| | | | Epimutations | | | | | | | | | | | | |
| | M | P | Epimutation | Origin | Epimutation | Origin | Epimutation | Origin | Epimutation | Origin | Epimutation | Origin | Epimutation | Origin | Epimutation | Origin | |
| 1 | c.2324C>T:p.P775L, rs542783229 | c.1267C>A:p.L423M | Hypermethylation | Germline, paternal allele | Hypomethylation | Germline or somatic, maternal allele | Hypomethylation | Somatic maternal allele | | | | | | | | | 4/7 |
| 3 | c.2426T>C:p.L809P, rs754189651 | c.655G>A:p.A219T | Hypermethylation | Germline, paternal allele | Hypermethylation | Somatic maternal allele | | | | | Hypomethylation | Somatic maternal allele | Hypomethylation | Somatic maternal allele | | | 4/7 |
| 5 | c.2396A>G:p.N799S, rs556133069 | c.1845A>C:p.Q615H | Hypermethylation | Germline, paternal allele | Hypomethylation | Somatic maternal allele | Hypomethylation | Somatic maternal allele | | | Hypomethylation | Somatic maternal allele | | | Hypomethylation | Somatic maternal allele | 5/7 |

Note: *M*, maternal allele; *P*, paternal allele

c.1267C>A:p.L423M and c.2324C>T:p.P775L. The first variant was located in exon 6 within the NACHT domain and was inherited from the father (Table 6, Fig. 2 and Suppl. Figure 4); it has not been previously reported. The second variant was located in the leucine-rich repeat (LRR) region and was inherited from the mother (Table 6 and Suppl. Figure 4). LRR domain-leucine-rich repeats are frequently involved in the formation of protein–protein interactions. The NACHT-associated domain is also the physical mediator of oligomeric assembly [26]. Nucleotide replacement at this position has been reported in normal populations (no data on reproductive disorders) and identified as rs542783229 (with a frequency of 0.01 (https://www.ensembl.org/Homo_sapiens/ Variation/Explore?db = core).

Miscarriage No 3. The gestational age at the time of this spontaneous abortion was 8 weeks of gestation according to ultrasound examination. Epimutations were found in imprinted genes expressed only from the paternal allele, including hypermethylation of *PEG1* and *PEG10* and hypomethylation of *KCNQ1OT1* and *PEG3* (Tables 6, 7 and Figs. 2, 4). The mother and father were 31 and 34 years old, respectively. The mother was diagnosed with RPL and had a history of three miscarriages at 6–8 weeks of gestation. Cytogenetic analysis showed a normal karyotype (46,XY). *NLRP7* gene sequencing revealed two heterozygous missense variants in the compound state: c.655G>A:p.A219T and c.2426T>C:p.L809P. Similar to miscarriage No 1, the first variant was located in exon 6 within the NACHT domain and was inherited from the father (Table 6, Figs. 2 and Suppl. Figure 5). This genetic variant is described here for the first time. The second variant was inherited from the mother; nucleotide replacement at this position was previously identified as rs754189651 (Table 6 and Suppl. Figure 5) and has been described in normal populations.

Miscarriage No 5. Embryonic development reached 7.5 weeks according to ultrasound examination. The mother and father were 34 and 39 years old, respectively. The mother showed RPL (three missed abortions in a period of 7–12 weeks). A normal karyotype (46,XY) was detected by the CGH and FISH methods, and somatic hypomethylation was identified at 4 imprinted genes (*PEG10*, *DLK1*, *KCNQ1OT1*, and *GRB10*) found only in one tissue. In contrast, *PEG1* showed germinal hypermethylation inherited from the father (Tables 6, 7 and Fig. 5). *NRLP7* gene sequencing showed two compound heterozygous variants: c.1845A>C:p.Q615H and c.2396A>G:p.N799S (Table 6 and Suppl. Figure 6). Both variants were located in LRR domains. The first variant was inherited from the mother and has previously

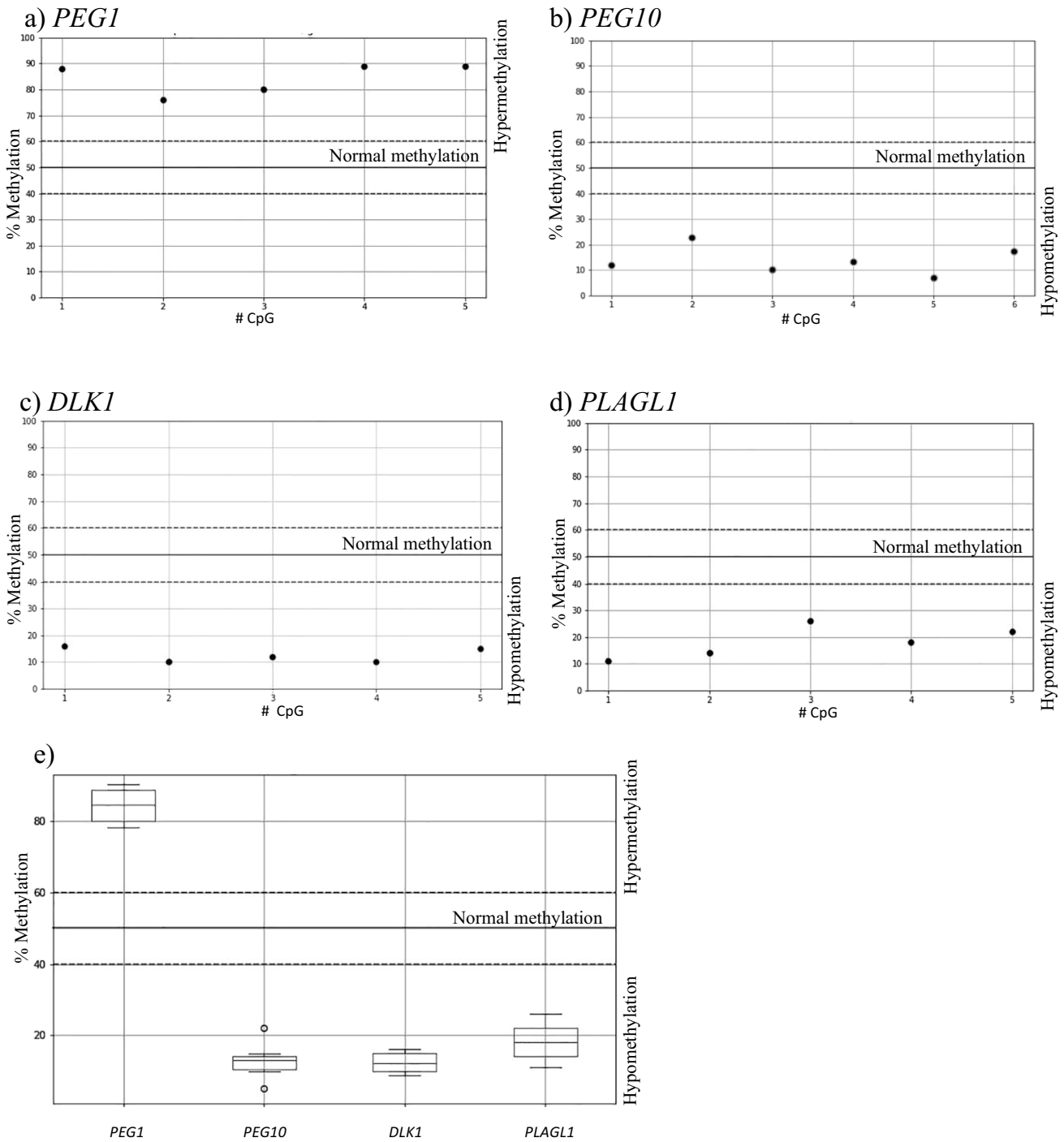


**Fig. 3** Methylation indices of imprinted genes in spontaneous abortion No. 1. Hypermethylation of *PEG1* (**a**); simultaneous hypomethylation of imprinted genes *PEG10* (**b**), *DLK1* (**c**), *PLAGL1* (**d**); and methylation of all studied imprinted loci (**e**)

been identified as rs556133069. The second variant was inherited from the father.

Thus, all NLRP7 variants detected in this study can change the conformation of the corresponding protein. Second, among the examined couples with RPL experiencing a miscarriage showing MLID for whom parental DNA was available, it was shown that both spouses harbored *NLRP7* gene variants in the heterozygous state. The inheritance of these variants in a compound heterozygous state can lead to prenatal developmental arrest and, thus, miscarriage. In addition, the *NLRP7* variants inherited from the father

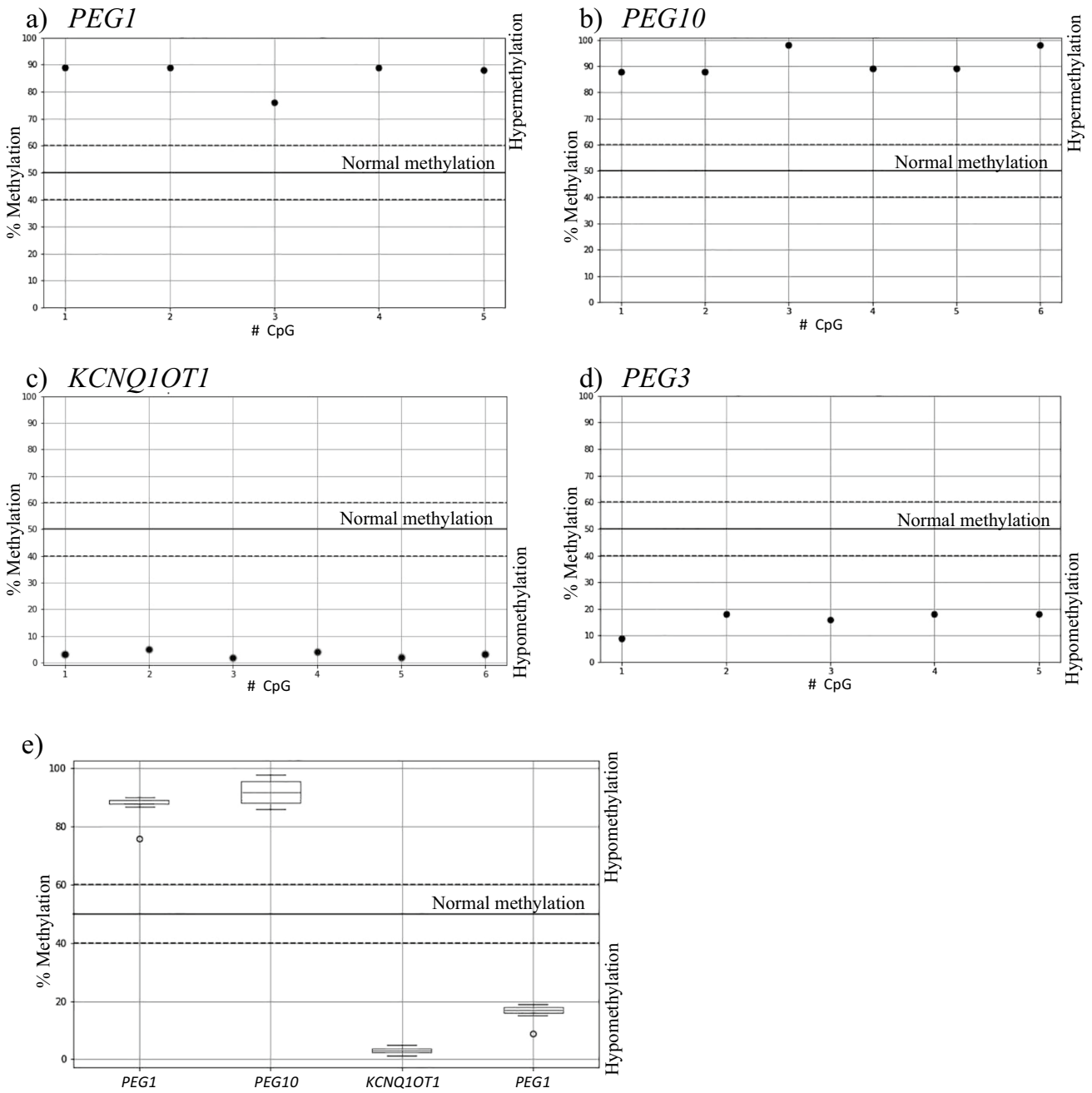


**Fig. 4** Methylation indices of imprinted genes in spontaneous abortion No. 3. Hypermethylation of the imprinted genes *PEG1* (**a**) and *PEG10* (**b**), hypomethylation of *KCNQ1OT1* (**c**) and *PEG3* (**d**), and methylation of all studied imprinted loci (**e**)

have not been previously described. This is in contrast to the mothers, in whom variants of this gene were described in databases recommended for the interpretation of NGS data. Third, germinal hypermethylation of paternal alleles was detected in the *PEG1* genes of three miscarriages. All of these findings suggest that the presence of *NLRP7* variants during both oogenesis and spermatogenesis can cause MLID in an embryo and disrupt prenatal development.

## Discussion

Genomic imprinting is an epigenetic phenomenon that causes monoallelic gene expression from one of the parental chromosomes. Imprinted genes play an important role in embryonic development. The result of epimutations in these genes is either doubled expression (upon hypomethylation) or a lack of expression (upon hypermethylation). In either case, this leads to a disturbance of the normal

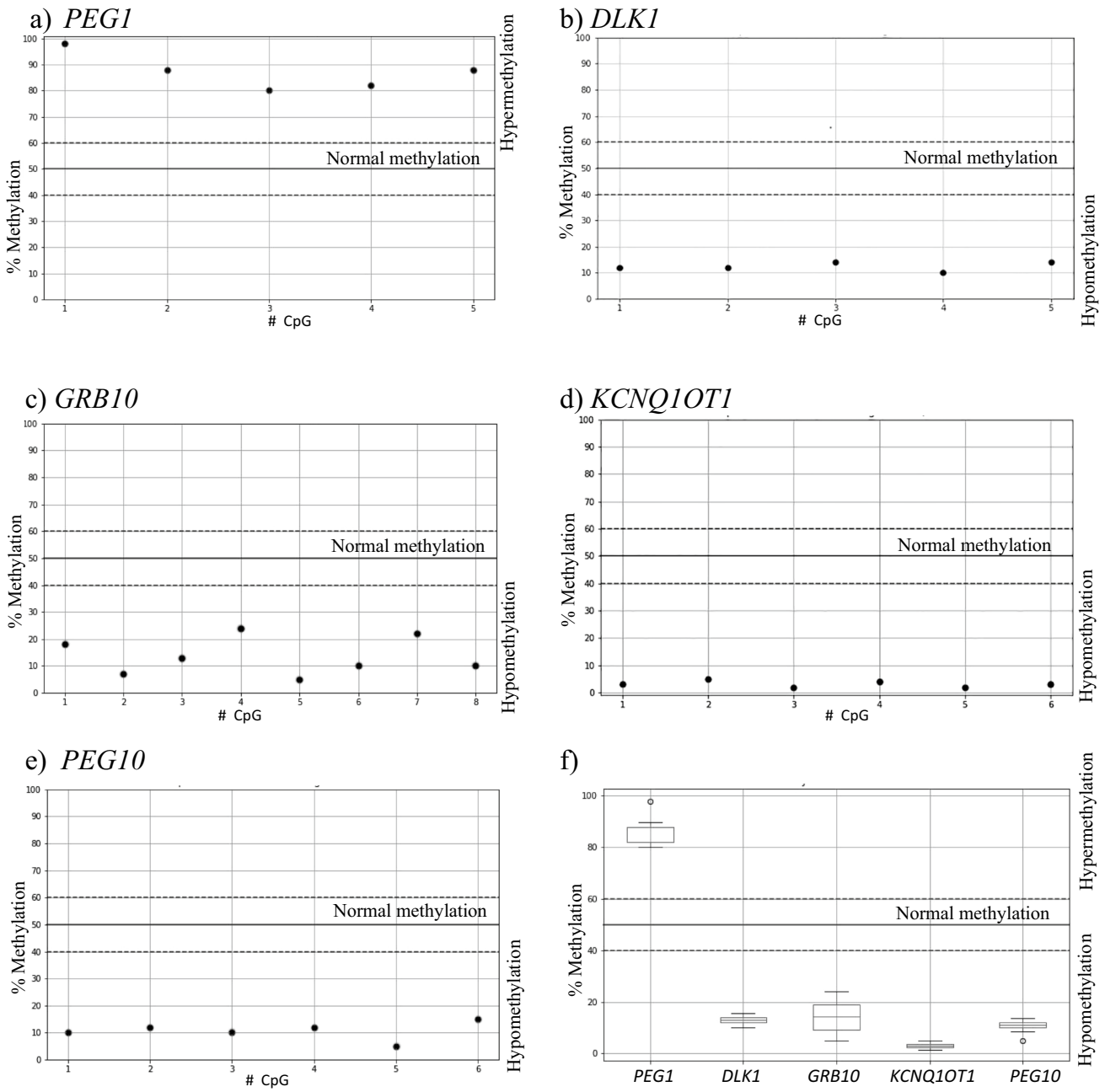


**Fig. 5** Methylation indices of imprinted genes in spontaneous abortion No. 5. Hypermethylation of the imprinted gene *PEG1* (**a**); hypomethylation of *DLK1* (**b**), *GRB10* (**c**), *KCNQ1OT1* (**d**), and *PEG10* (**e**); and methylation of all studied imprinted loci (**f**)

function of the imprinted genes. In this study, we showed that epimutations contributed to the arrest of embryonic development and were found at a higher frequency in miscarriages from the group of women with RPL than in miscarriages from the group of women with SPL (7.1% and 2.7%, respectively, $p < 0.001$).

The frequency of MLID was also higher among miscarriages of women with RPL than among those of women with SPL (1.72% and 0.37%, respectively, $p = 0.0001$). The reported frequency of MLID among spontaneous abortions is approximately 3.6% according to other authors [12, 13]. This is consistent with our data. Approximately 20% of all clinically diagnosed pregnancies end in spontaneous abortion, among which 75–80% end within 12 weeks of gestation. Half of these miscarriages have a normal karyotype [1]. Therefore, the population frequency of MLID among miscarriages was approximately 0.16% in the RPL group and 0.035% in the SPL group. MLID is also observed in live-born individuals with SRS and BWS, as are (less frequently) transient neonatal diabetes mellitus and sporadic

pseudoparathyroidism 1B. The population frequencies of MLID for SRS and BWS cases are 1:263,158 (0.0004%) and 1:109,600 (0.0009%), respectively [15]. These frequencies were significantly lower than the frequency of MLID among miscarriages (0.16% in the RPL group and 0.035% in the SPL group in our study). Thus, natural selection acts against MLID during prenatal development.

Several hypotheses have been proposed to explain the appearance of genomic imprinting in evolution, the best supported of which is Haig's "sex conflict hypothesis" [27]. Under this hypothesis, functional haploidization of imprinted sites in the genome limits the involvement of maternal genes and increases the role of paternal genes in the formation of placental structures. Indeed, we observed the predominant hypomethylation of maternal alleles and hypermethylation of paternal alleles among placental tissues from miscarriages of women with RPL in the present study. According to the "sex conflict hypothesis", such epigenetic disturbances could result in an increase in suppressors and a decrease in factors stimulating embryonic development.

MILD indicates the possibility of the existence of regulatory mechanisms controlling the epigenetic status of multiple imprinted loci. Therefore, we hypothesize that the presence of genetic variants in *NLRP7* among miscarriages may be the cause of MLIDs, which are responsible for the regular arrest of embryonic development without BiHM. In our study, the results of *NLRP7* sequencing revealed nucleotide variants in 7 miscarriages, only from women with RPL. Among all of the spontaneous abortions with variants in *NLRP7*, epimutations were found in at least three imprinted genes, taking the form of either hypomethylation or hypermethylation. The present findings support our hypothesis that the presence of *NLRP7* variants in the heterozygous state in both spouses can lead to the inheritance of the compound heterozygous state in an embryo. This leads to multiple epimutations at imprinted loci and consequently the arrest of embryonic development. In addition, the genetic variants identified in all analyzed miscarriages that were inherited from the fathers were not previously detected, whereas all identified known variants had a maternal origin. Three miscarriages taken in the analysis also showed germinal hypermethylation of paternal alleles of the *PEG1* gene. All of these findings suggest that the existence of *NLRP7* variants during both oogenesis and spermatogenesis can affect the epigenetic status of imprinted loci in the embryo and lead to the arrest of embryonic development. Thus, genetic variants in this gene can not only exert an indirect influence through the maternal genome but can also directly disrupt methylation at several imprinted loci in the embryo itself. Previous research has established an association between homozygous or compound-heterozygous maternal effect variants in the *NLRP7* gene and recurrent diploid BiHM [28, 29]. In contrast, the present findings support the hypothesis that heterozygous *NLRP7* variants from parental carriers result in an increased risk of reproductive wastage without the BiHM phenotype. The identification of heterozygous variants in *NLRP7* has been documented in other reports (in which the presence of *NLRP7* gene variants was not examined in the father or fetus) [30, 31]. The women included in these studies had a history of miscarriages with MLID and embryonic development arrest. These data support our hypothesis that parental heterozygous *NLRP7* variants are associated with reproductive problems without BiHM in a patient's medical history and lead to MLIDs in the offspring and reduced embryonic survival. Thus, it makes sense to search for mutations in the *NLRP7* gene in both spouses if a woman has RPL without the BiHM phenotype.

*NLRP7* (*NALP7*, *NALP7*/*PYPAF3*, OMIM 609661, locus 19q13.42) contains PYD, NACHT-NAD, and LRR repeats. NLRP7 is a member of the CATERPILLAR protein family, a negative regulator of inflammation and apoptosis, and an inhibitor of the secretion of interleukin 1-beta mediated by caspase-1 activation. *NLRP7* is expressed in all human tissues except for the heart, brain, and skeletal muscle. There is no ortholog of human *NLRP7* in the mouse genome, and it is thought to have originated via the duplication of *Nlrp2* during evolution. Using RNA interference, it was found that the knockdown of *Nlrp2* in mice does not affect oocyte maturation but leads to the arrest of embryonic development at the two-cell stage [32]. In humans, genetic variants in *NLRP2* accompanied by MLID have been associated with BWS [33] and idiopathic recurrent miscarriage [34]. The *NLRP7* sequence is rich in Alu repeats, which account for 48% of intron regions and can lead to Alu-mediated deletions and rearrangements within a gene, which occur 180 times more often than all other known genetic variants in humans on average. These repeats were probably embedded in the *Nlrp2*/*7* gene of the Primate ancestor. Such variants in *NLRP7* can lead to miscarriage in women [35].

Deveault and coauthors found that patients with *NLRP7* genetic variants were unable to establish an appropriate inflammatory response to different antigens [36]. Consequently, androgenetic blastocysts consisting completely of allograft material can be implanted and develop without being rejected in such women. It is possible that androgenetic blastocysts form spontaneously *de novo* with some frequency. However, in women with an active immune system, androgenetic cells are more likely to die or stop developing and go undetected.

Furthermore, Okada found that the knockdown of *NLRP7* by RNA interference resulted in reduced growth of a carcinoma cell line, indicating that this gene may play a crucial role in cell proliferation [37]. In addition, the NLRP7 protein can directly affect the methylation status of imprinted

regions by binding to the YY1 epitope of transcription factors in imprinted differentially methylated regions [38].

Interestingly, the *NLRP7* gene product is included in SCMC. During the period of follicular development in mammals, oocytes accumulate significant amounts of maternal RNAs and proteins that are needed before the beginning of zygotic genome activation. This activation occurs at the two-cell stage in mice and the 4–8-cell stage in humans. In addition to NLRP7, SCMC includes the KHDC3L (mouse homolog, Filia), NLRP5 (mouse homolog, Mater), OOEP (a factor located in oocytes allowing embryonic development (mouse homolog Floped)), and Tle6 proteins. The expression of the genes encoding this protein complex is necessary for oocytes and early mammalian embryos to develop a zygote at the first cell divisions [39–41]. Within this large complex, Floped, Mater, and Tle6 interact with each other, and Filia independently binds to Mater. Although the transcripts encoding these proteins degrade during oocyte maturation and ovulation, SCMC proteins persist in the early embryo until the blastocyst stage. Mating, oogenesis, ovulation, and fertilization in Filia-null female mice are normal, but their fertility is reduced by approximately 50%, which is associated with a delay in embryonic development. Embryos exhibit hyperploidy because of the asymmetric cell division during meiosis. The null variant of the *Mater*, *Floped*, or *Tle6* gene in mice leads to embryonic developmental arrest and female infertility [40]. We have shown that *NLRP7 v*ariants affect the LRR and NACHT domains, which are involved in the formation of protein–protein interactions or the physical mediation of oligomeric assembly [26]. Imprinted genes are mainly protein-coding genes and are important for embryo development. In the studied miscarriages, it is possible that *NLRP7* variants lead to the accumulation of MLID in the embryo, which disrupts the normal monoallelic expression of many imprinted genes and leads to disruption of embryonic development during the first trimester of pregnancy rather than in the first stages of preimplantation development.

In most studies conducted to date, only the maternal effect of *NLRP7* variants has been investigated [42], and the paternal contribution to the disturbance of embryonic development has been considered insignificant. In the present study, we show that not only maternal but also paternal *NLRP7* contributes to normal embryonic development.

From a practical point of view, two diagnostic strategies are possible for families with a history of RPL. The first is direct sequencing of the *NLRP7* gene in both the mother and father to detect genetic variants that may be associated with abnormalities in imprinted gene methylation and pregnancy loss. The second strategy can be applied if the placental or embryo tissues of miscarriage are available for DNA methylation analysis of the imprinted genes. In this case, preliminary detection of MLID will increase the chance of finding *NLRP7* variants with pathogenic or potentially pathogenic significance in embryos as well as in maternal or paternal genomes.

In summary, we detected variants in the *NLRP7* gene only among miscarriages with MLIDs of imprinted genes from women with recurrent miscarriages, which suggests that carrying these genetic variants in a heterozygous state at parents can be considered a risk factor for RPL.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10815-021-02312-z.

**Acknowledgements** Molecular cytogenetic and molecular genetic studies were performed in the "Medical Genomics" Core Facility of the Tomsk National Research Medical Center of the Russian Academy of Sciences using the resources of the "Biobank of the Population of Northern Eurasia" biocollection of the Research Institute of Medical Genetics, Tomsk NRMC. We would like to thank all the families for their participation in the study. We are grateful to all the clinicians who were involved in sample collection.

**Author contribution** EAS, VAS and INL conceived the study and prepared the manuscript. TVN performed the cytogenetic analyses of spontaneous abortions. EAS and ENT performed methyl-specific and methyl-sensitive PCR as well as methylation-specific pyrosequencing of imprinted genes. OYuV prepared DNA libraries and performed massively parallel sequencing and Sanger sequencing. NAK performed the identified *NLRP7* variants from our whole genome data on 700 population samples assessed. SAV, AAZ and AVM performed statistical and bioinformatic analyses of the obtained results. SYuY was involved in the organization of sample collection. All of the authors have read and approved the manuscript.

**Funding** The study was supported by the Ministry of Science and Education of the Russian Federation (budgetary project of the Research Institute of Medical Genetics, TNRMC, # AAAA-A19-11902089005–5).

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

**Code availability** Not applicable.

## Declarations

**Ethics approval and consent to participate** All procedures performed in studies involving human participants were in accordance with the ethical standards of the Scientific Ethics Committee of the Research Institute of Medical Genetics of the Tomsk NRMC (Protocol # 6 from 08.11.2012) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

**Consent for publication** The authors have read and approved the manuscript and agree with its submission to the Journal of Assisted Reproduction and Genetics.

**Conflict of interest** The authors declare that they have no conflicts of interest that could be perceived as prejudicing the impartiality of this manuscript.

## References


1. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil Steril. 2020;113:533–5. https://doi.org/10.1016/j.fertnstert.2019.11.025
2. Nikitina TV, Sazhenova EA, Zhigalina DI, Tolmacheva EN, Sukhanova NN, Lebedev IN. Karyotype evaluation of repeated abortions in primary and secondary recurrent pregnancy loss. J Assist Reprod Genet. 2020;37:517–25. https://doi.org/10.1007/s10815-020-01703-y.
3. Tucci V, Isles AR, Kelsey G, Ferguson-Smith AC, Tucci V, Bartolomei MS, Benvenisty N. Genomic imprinting and physiological processes in mammals. Cell. 2019;176:952–65. https://doi.org/10.1016/j.cell.2019.01.043.
4. Nikitina TV, Sazhenova EA, Sukhanova NN, Lebedev IN, Nazarenko SA. Evaluation of the role of uniparental disomy in early embryolethality of man. Ontogenez. 2004;35:297–306. https://doi.org/10.1023/B:RUDO.0000036715.98103.fc.
5. Kondo Y, Tsukishiro S, Tanemura M, Sugiura-Ogasawara M, Suzumori K, Sonta SI. Maternal uniparental disomy of chromosome 16 in a case of spontaneous abortion. J Hum Genet. 2004;49:177–81. https://doi.org/10.1007/s10038-004-0128-5.
6. Tsukishiro S, Li QY, Tanemura M, Sugiura-Ogasawara M, Suzumori K, Sonta SI. Paternal uniparental disomy of chromosome 14 and unique exchange of chromosome 7 in cases of spontaneous abortion. J Hum Genet. 2005;50:112–7. https://doi.org/10.1007/s10038-005-0229-9.
7. Sazhenova EA, Lebedev IN. Evolutionary aspects of genomic imprinting. Mol Biol. 2021;55:1–15. https://doi.org/10.1134/s0026893320060102.
8. Sazhenova EA, Lebedev IN. Epimutations of the KCNQ1OT1 imprinting center of chromosome 11 in early human embryolethality. Russ J Genet. 2008;44:1394–9. https://doi.org/10.1134/s1022795408120028.
9. Sazhenova EA, Lebedev IN. Epimutations of the PLAGL1 imprinted gene in patients with recurrent miscarriage. Med Genet. 2010;9:34–9.
10. Sazhenova EA, Skryabin NA, Sukhanova NN, Lebedev IN. Multilocus epimutations of imprintome in the pathology of human embryo development. Mol Biol. 2012;46:204–13. https://doi.org/10.1134/S0026893312010207.
11. Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur JG, Chen J. Differential expression of imprinted genes in normal and IUGR human placentas. Epigenetics. 2009;4:235–40. https://doi.org/10.4161/epi.9019.
12. Pliushch G, Schneider E, Weise D, El Hajj N, Tresch A, Seidmann L, Coerdt W, Müller AM, Zechner U, Haaf T. Extreme methylation values of imprinted genes in human abortions and stillbirths. Am J Pathol. 2010;176:1084–90. https://doi.org/10.2353/ajpath.2010.090764.
13. Zechner U, Pliushch G, Schneider E, El Hajj N, Tresch A, Shufaro Y, Seidmann L, Coerdt W, Muller AM, Haaf T. Quantitative methylation analysis of developmentally important genes in human pregnancy losses after ART and spontaneous conception. Mol Hum Reprod. 2010;16:704–13. https://doi.org/10.1093/molehr/gap107.
14. Zheng HY, Tang Y, Niu J, Li P, Ye DS, Chen X, Shi XY, Li L, Chen SL. Aberrant DNA methylation of imprinted loci in human spontaneous abortions after assisted reproduction techniques and natural conception. Hum Reprod. 2012;28:265–73. https://doi.org/10.1093/humrep/des358.
15. Soellner L, Begemann M, Mackay DJG, Grønskov K, Tümer Z, Maher ER, Temple IK, Monk D, Riccio A, Linglart A, Netchine I, Eggermann T. Recent advances in imprinting disorders. Clin Genet. 2016;91:3–13. https://doi.org/10.1111/cge.12827.
16. Elbracht M, Mackay D, Begemann M, Kagan KO, Eggermann T. Disturbed genomic imprinting and its relevance for human reproduction: causes and clinical consequences. Hum Reprod Update. 2020;26:197–213. https://doi.org/10.1093/humupd/dmz045.
17. Kalogiannidis I, Kalinderi K, Kalinderis M, Miliaras D, Tarlatzis B, Athanasiadis A. Recurrent complete hydatidiform mole: where we are, is there a safe gestational horizon? Opinion and mini-review. J Assist Reprod Genet. 2018;35:967–73. https://doi.org/10.1007/s10815-018-1202-9.
18. Lu X, Gao Z, Qin D, Li L. A maternal functional module in the mammalian oocyte-to-embryo transition. Trends Mol Med. 2017;23:1014–23. https://doi.org/10.1016/j.molmed.2017.09.004.
19. Ostroverkhova NV, Nazarenko SA, Lebedev IN, Cheremnykh AD, Nikitina TV, Sukhanova NN. Detection of aneuploidy in spontaneous abortions using the comparative hybridization method. Russ J Genet. 2002;38:1690–8. https://doi.org/10.1023/A:1021604325687.
20. Lebedev IN, Ostroverkhova NV, Nikitina TV, Sukhanova NN, Nazarenko SA. Features of chromosomal abnormalities in spontaneous abortion cell culture failures detected by interphase FISH analysis. Eur J Hum Genet. 2004;12:513–20. https://doi.org/10.1038/sj.ejhg.5201178.
21. Simoni G, Brambati B, Danesino C, Rossella F, Terzoli GL, Ferrari M, Fraccaro M. Efficient direct chromosome analyses and enzyme determinations from chorionic villi samples in the first trimester of pregnancy. Hum Genet. 1983;63:349–57. https://doi.org/10.1007/bf00274761.
22. Sazhenova EA, Nikitina TV, Skryabin NA, Minaycheva LI, Ivanova TV, Nemtseva TN, Yuriev SY, Evtushenko ID, Lebedev IN. Epigenetic status of imprinted genes in placenta during recurrent pregnancy loss. Russ J Genet. 2017;53:376–87. https://doi.org/10.1134/s1022795417020090.
23. Monk D, Morales J, den Dunnen JT, Russo S, Court F, Prawitt D, Eggermann T, Beygo J, Buiting K, Tümer Z & the Nomenclature group of the European Network for Human Congenital Imprinting Disorders. Recommendations for a nomenclature system for reporting methylation aberrations in imprinted domains// Epigenetics. 2018; 13;2:117–121. https://doi.org/10.1080/15592294.2016.1264561
24. den Dunnen JT, Dalgleish R, Maglott DR, Hart RK, Greenblatt MS, McGowan-Jordan J, Roux A-F, Smith T, Antonarakis SE, Taschner PEM on behalf of the Human Genome Variation Society (HGVS), the Human Variome Project (HVP), and the Human Genome Organization (HUGO) HGVS Recommendations for the Description of Sequence Variants: 2016 Update// Human Mutation. 2016;37;6:564–569. https://doi.org/10.1002/humu.22981
25. Richards S, Aziz N, Bale S., Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL on behalf of the ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology// Genetics in Medicine. 2015; 17;5: 405–424. 5. https://doi.org/10.1038/gim.2015.30
26. Singer H, Biswas A, Zimmer N, Messaed C, Oldenburg J, Slim R, El-Maarri O. *NLRP7* inter-domain interactions: the NACHT-associated domain is the physical mediator for oligomeric assembly// Mol Hum Reprod. 2014; 20;10: 990–1001. https://doi.org/10.1093/molehr/gau060
27. Haig D. The Kinship theory of genomic imprinting. Annu Rev Ecol Syst. 2000;31:9–32. https://doi.org/10.1146/annurev.ecolsys.31.1.9.

28. Nguyen NMP, Khawajkie Y, Mechtouf N, Rezaei M, Breguet M, Kurvinen E, Jagadeesh S, Solmaz AE, Aguinaga M, Hemida R, Harma MI, Rittore C, Rahimi K, Arseneau J, Hovanes K, Clisham R, Lenzi T, Scurry B, Addor M-C, Bagga R, Nendaz GG, Finci V, Poke G, Grimes L, Gregersen N, York K, Bolze P-A, Patel C, Mozdarani H, Puechberty J, Scotchie J, Fardaei M, Harma M, Gardner RJM, Sahoo T, Dudding-Byth T, Srinivasan R, Sauthier P, Slim R. The genetics of recurrent hydatidiform moles: new insights and lessons from a comprehensive analysis of 113 patients. Mod Pathol. 2018;31:1116–30. https://doi.org/10.1038/s41379-018-0031-9.
29. Butt SA, Kelstrup L, Lidang M, Bertelsen M, Ejrnæs K, Sunde L, Perin TL. Recurrent diploid biparental mole.Ugeskr Laeger. 2019;181:V02190144
30. Messaed C, Chebaro W, Roberto RBD, Rittore C, Cheung A, Arseneau J, Schneider A, Chen MF, Bernishke K, Surti U, Hoffner L, Sauthier P, Buckett W, Qian J, Lau NM, Bagga R, Engert JC, Coullin P, Touitou I, Slim R. NLRP7 in the spectrum of reproductive wastage: rare non-synonymous variants confer genetic susceptibility to recurrent reproductive wastage. J Med Genet. 2011;48:540–8. https://doi.org/10.1136/jmg.2011.089144.
31. Soellner L, Begemann M, Degenhardt F, Geipel A, Eggermann T, Mangold E. Maternal heterozygous *NLRP7* variant results in recurrent reproductive failure and imprinting disturbances in the offspring. Eur J Hum Genet. 2017;25:924–9. https://doi.org/10.1038/ejhg.2017.94.
32. Peng H, Chang B, Lu C, Su J, Wu Y, Lv P, Wang Y, Liu J, Zhang B, Quan F, Guo Z, Zhang Y. *Nlrp2*, a maternal effect gene required for early embryonic development in the mouse. PLoS ONE. 2012;7: e30344. https://doi.org/10.1371/journal.pone.0030344.
33. Meyer E, Lim D, Pasha S, Tee LJ, Rahman F, Yates JRW, Woods CG, Reik W, Maher ER. Germline mutation in NLRP2 (NALP2) in a familial imprinting disorder (Beckwith-Wiedemann Syndrome). PLoS Genet. 2009;5: e1000423. https://doi.org/10.1371/journal.pgen.1000423.
34. Huang JY, Su M, Lin SH, Kuo PL. A genetic association study of *NLRP2* and *NLRP7* genes in idiopathic recurrent miscarriage. Hum Reprod. 2013;28:1127–34. https://doi.org/10.1093/humrep/det001.
35. Reddy R, Akoury E, Nguyen NMP, Abdul-Rahman OA, Dery C, Gupta N, Daley WP, Ao A, Landolsi H, Fisher RA, Touitou I, Slim R. Report of four new patients with protein-truncating mutations in C6orf221/*KHDC3L* and colocalization with *NLRP7*. Eur J Hum Genet. 2013;21:957–64. https://doi.org/10.1038/ejhg.2012.274.
36. Deveault C, Qian JH, Chebaro W, Ao A, Gilbert L, Mehio A, Khan R, Tan SL, Wischmeijer A, Coullin P, Xie X, Slim R. *NLRP7* mutations in women with diploid androgenetic and triploid moles: a proposed mechanism for mole formation. Hum Mol Genet. 2009;18:888–97. https://doi.org/10.1093/hmg/ddn418.
37. Okada K, Hirota E, Mizutani Y, Fujioka T, Shuin T, Miki T, Nakamura Y, Katagiri T. Oncogenic role of NALP7 in testicular seminomas. Cancer Sci. 2004;95:949–54. https://doi.org/10.1111/j.1349-7006.2004.tb03182.x.
38. Mahadevan S, Wen S, Wan YW, Peng HH, Otta S, Liu Z, Iacovino M, Mahen EM, Kyba M, Sadikovic B, Van den Veyver IB. NLRP7 affects trophoblast lineage differentiation, binds to overexpressed YY1 and alters CpG methylation. Hum Mol Genet. 2014;23:706–16. https://doi.org/10.1093/hmg/ddt457.
39. Zhu K, Yan L, Zhang X, Lu X, Wang T, Yan J, Liu X, Qiao J, Li L. Identification of a human subcortical maternal complex. Mol Hum Reprod. 2014;21:320–9. https://doi.org/10.1093/molehr/gau116.
40. Bebbere D, Masala L, Albertini DF, Ledda S. The subcortical maternal complex: multiple functions for one biological structure? J Assist Reprod Genet. 2016;33:1431–8. https://doi.org/10.1007/s10815-016-0788-z.
41. Monk D, Sanchez-Delgado M, Fisher R. NLRPs, the subcortical maternal complex and genomic imprinting. Reproduction. 2017;154:R161–70. https://doi.org/10.1530/rep-17-0465.
42. Begemann M, Rezwan FI, Beygo J, Docherty LE, Kolarova J, Schroeder C, Buiting K, Chokkalingam K, Degenhardt F, Wakeling EL, Kleinle S, Fassrainer DG, Oehl-Jaschkowitz B, Turner CLS, Patalan M, Gizewska M, Binder G, Bich Ngoc CT, Chi Dung V, Mehta SG, Baynam G, Hamilton-Shield JP, Aljareh S, Lokulo-Sodipe O, Horton R, Siebert R, Elbracht M, Temple IK, Eggermann T, Mackay DJG. Maternal variants in NLRP and other maternal effect proteins are associated with multilocus imprinting disturbance in offspring. J Med Genet. 2018;55:497–504. https://doi.org/10.1136/jmedgenet-2017-105190.