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HUMAN GENETICS

Methylation of the Retrotransposon LINE-1 Subfamilies in Chorionic Villi of Miscarriages

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Abstract—Miscarriage is potentially associated with abnormal epigenetic regulation of genes responsible for the development of the embryo and placenta. The aim of this work was to analyze the methylation level of various subfamilies of the LINE-1 retrotransposon, which makes up about 17% of the entire genome, in chorionic villi of spontaneous abortions of the first trimester of pregnancy with different karyotypes, including the most common aneuploidies. The methylation profile in the LINE-1 retrotransposon promoter was analyzed using targeted bisulfite massive parallel sequencing in chorionic villi of induced abortions (n = 39), spontaneous abortions with normal karyotype (n = 173), trisomy 16 (n = 62) and monosomy X (n = 46), and peripheral blood lymphocytes of healthy volunteers (n = 17). The level of methylation of the LINE-1 retrotransposon subfamilies in the control groups of adult lymphocytes and chorionic villi of induced abortions was the highest for evolutionarily young L1HS subfamilies, lower for the more ancient L1PA2 and L1PA3 subfamilies, and the lowest for the even more ancient L1PA4 subfamily. In the groups of spontaneous abortions, an increased level of LINE-1 methylation was observed, and this effect was more pronounced for the older LINE-1 subfamilies. The revealed patterns indicate less control over the older subfamilies of the LINE-1 retrotransposon in the human genome, which can potentially be used as regulatory elements for nearby genes involved in embryonic development. An increase in the level of methylation of such sequences can disrupt the development of the placenta and embryo and make a certain contribution to miscarriage.

Keywords: LINE-1 retrotransposon, DNA methylation, chorionic villi, miscarriage, aneuploidy, bisulfite sequencing, spontaneous abortions

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INTRODUCTION

Miscarriage is common, affecting about 15% of all pregnancies [1]. There are many known causes of miscarriage, the main one being aneuploidy. However, almost 50% of early pregnancy losses remain unexplained [2].

The placenta plays a leading role in early embryonic development, providing nourishment to the fetus and interaction between it and the mother. By the end of the first trimester of pregnancy, the normal course of the process of remodeling of the spiral arteries becomes critical for the further development of the embryo, since until the ninth week of pregnancy, plugs from cells of the extravillous trophoblast limit the flow of maternal blood into the space between the chorionic villi. Occlusion helps maintain a state of physiological hypoxia during the early stages of the placentation process by promoting cytotrophoblast proliferation rather than differentiation and invasion [3]. After the disintegration of these plugs at approximately the ninth week of pregnancy, the uteroplacental spiral arteries begin to supply maternal blood to the intervillous space [4]. Embryos that cannot ensure the normal course of this process, apparently die because of hypoxia and lack of nutrients.

Epigenetic mechanisms regulating gene expression play a fundamental role in controlling placental development [5]. The placenta has a specific methylome that significantly distinguishes it from embryonic and adult tissues [6]. Its key property is the low level of methylation of various repeating sequences, including endogenous retroviruses and retrotransposons [7]. It is known that genes of retroviral origin play an important role in the formation and functioning of the placenta [8]. Errors in the regulation of such sequences, mainly due to DNA methylation, could potentially explain placental dysfunction in early pregnancy and lead to miscarriage.

In addition, various mobile genetic elements, including endogenous human retroviruses and retro-

transposons, play an important role in the early stages of embryonic and placental development [9, 10]. LINE-1 subfamilies differ from each other in individual nucleotide substitutions that are specific to all members of a particular subfamily. Five subfamilies of the LINE-1 retrotransposon (L1P1, L1PA2, L1PA3, L1PA4, L1PA5) have proliferated in the genomes of hominoid primates [11, 12]. Another subfamily (L1HS) is specific only to humans [13]. Previously, we identified disturbances in the level of methylation of the retrotransposon LINE-1 in the chorionic villi of spontaneous abortions in the first trimester of pregnancy with aneuploidy on various chromosomes [14].

The aim of this study was to analyze the level of methylation of various subfamilies of the LINE-1 retrotransposon in the chorionic villi of spontaneous abortions of the first trimester of pregnancy with a normal karyotype and with the most frequent aneuploidies—trisomy 16 and monosomy X.

MATERIALS AND METHODS

As material for the study, samples of chorionic villi were sampled from induced abortions (IA) (n = 39, gestational age 8.3 ± 1.8 weeks), spontaneous abortions (SA) with a normal karyotype (n = 173, gestational age 7.6 ± 1.9 weeks), trisomy 16 (n = 62, gestational age 6.9 ± 1.9 weeks), and monosomy X (n = 46, gestational age 8.8 ± 1.2 weeks). Samples taken from the Biobank of the population of Northern Eurasia of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center were obtained in the period from 1993 to 2022. Prior to the start of the study, the samples were stored at −80°C without thawing. Peripheral blood lymphocytes from healthy volunteers were used as a comparison group (n = 17, age 30.8 ± 2.7 years). The study was conducted ethically in accordance with the Declaration of Helsinki of the World Medical Association. For all samples from the biobank, informed consent was obtained from the parents to use the biomaterial for biobanking and research. Informed consents were also obtained from healthy volunteers. The study was approved by the Biomedical Ethics Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center (November 9, 2020/no. 7).

To determine the karyotype, a standard cytogenetic analysis was conducted on direct preparations of chorionic villi and cultures of fibroblasts of the extraembryonic mesoderm [15]. The results of karyotyping of all spontaneous abortions with aneuploidy were confirmed by fluorescence in situ hybridization (FISH). The level of mosaicism of aneuploidy was assessed with a lower threshold of 10% and an upper threshold of 90%. For the analysis of monosomy on the X chromosome, centromere-specific DNA probes for the X chromosome were used, and for the analysis of trisomy on the chromosome 16, subtelomeric DNA probes (16q and 16p) were used. The analysis was carried out according to the previously described method [16]. Genomic DNA isolation from chorionic villi and peripheral blood lymphocytes was performed using the phenol-chloroform method.

Primers for targeted enrichment of the promoter portion of the LINE-1 retrotransposon using PCR were designed on the basis of the reference sequence of the LINE-1 retrotransposon subfamily L1HS taken from the GenBank database (X58075.1). The analysis was carried out in accordance with a previously developed protocol [17].

Targeted bisulfite massive parallel sequencing was carried out on a MiSeq instrument (Illumina, United States) using a Nano Kit (2 × 250). Read quality assessment was performed using FastQC v0.11.8, after which the remaining adapter sequences and low-quality reads were trimmed using Trim-Galore. Reads were then mapped to bisulfite-converted target sequences using the bwa-meth v0.2.2 tool (https://github.com/brentp/bwa-meth) with default parameters. Methylation data in the context of CpG were extracted from the resulting BAM files using the MethylDackel tool (https://github.com/ dpryan79/MethylDackel). The level of methylation was analyzed only in CpG sites with a coverage greater than 10.

The annealing sites of the primers used are present in the sequences of several LINE-1 subfamilies. BLAST analysis was performed to determine the level of identity (https://blast.ncbi.nlm.nih.gov/) using the bisulfite-converted sequence of the LINE-1 promoter region, obtained using the primers developed in this work, and bisulfite-converted consensus sequences of repeats in the human genome, obtained from the UCSC Repeat Browser [18] (Fig. 1). The results of the analysis showed that the L1P2, L1PA6, LTR65, HERVL18 and MER110-int sequences matching L1HS lack a forward primer sequence, and the L1PA7 and L1PA10 sequences lack a reverse primer sequence. Therefore, no PCR product could be obtained for these subfamilies using the designed primers. As a result, complete sequence overlap was found only for the following LINE-1 subfamilies: L1HS, L1P1, L1PA2, L1PA3, L1PA4, and L1PA5. Three regions with increased variability without CpG sites were identified, the SNPs in which can be used for consistent classification of reads belonging to different LINE-1 subfamilies (Fig. 1). The first region allows one to filter the L1HS and L1PA2 subfamilies; the second region, the L1PA5 subfamilies; the third region, the L1PA3, L1PA4, and L1P1 subfamilies. For each unassigned read, a BLAST analysis was performed against the LINE-1 subfamily consensus sequences, which assigned the read to the subfamily with the highest degree of identity. When making comparisons, the positions of cytosines were masked.

The results were presented as the methylation level, which is the ratio of the number of cytosines to the total number of cytosines and thymines in an individual CpG site. In addition, the average methylation level along the entire region of interest was calculated.

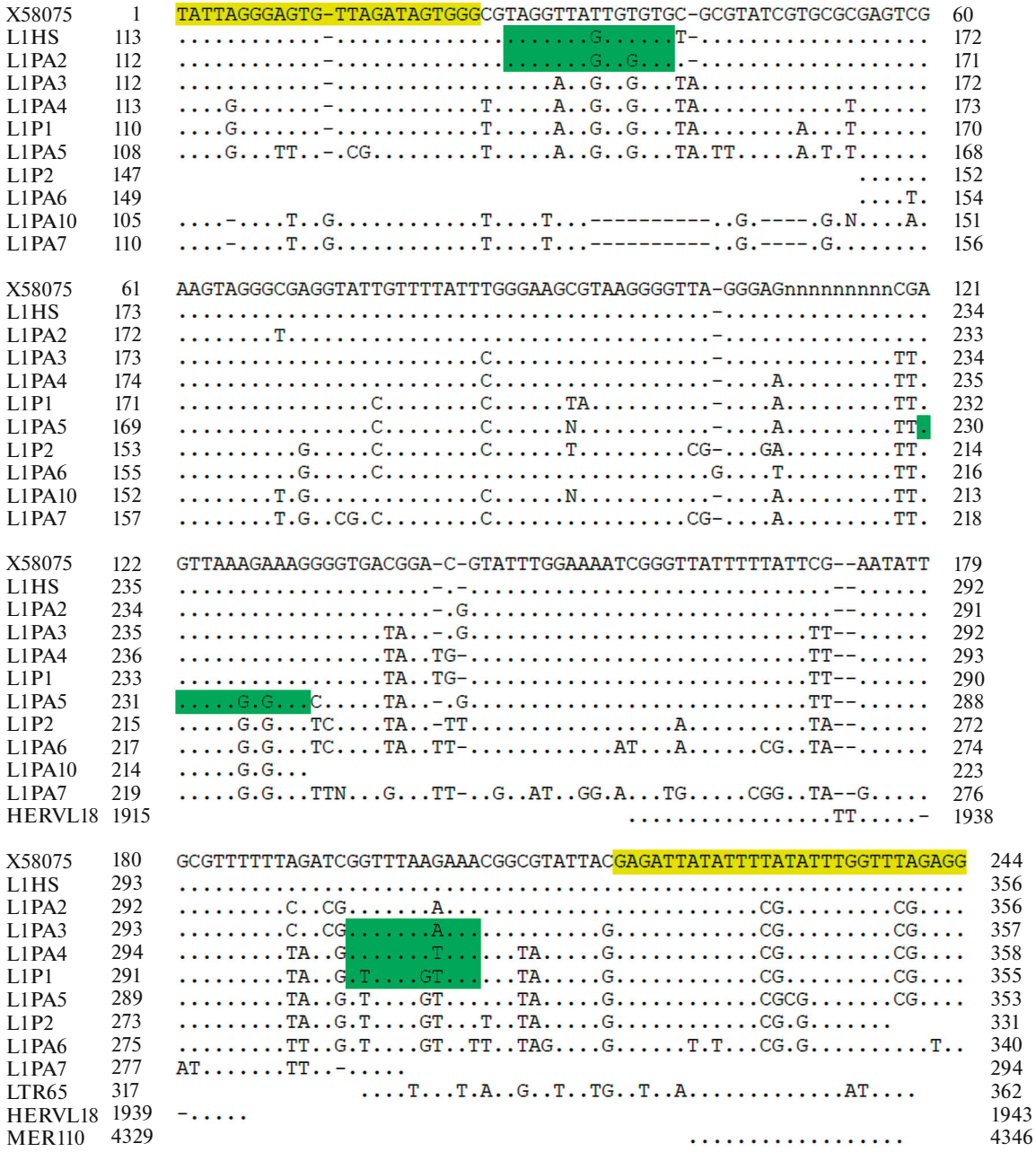

Fig. 1. Results of BLAST analysis of the bisulfite-converted sequence of the LINE-1 promoter region, obtained using the primers developed in this work, and bisulfite-converted consensus sequences of repeats in the human genome, obtained from the UCSC Repeat Browser. The top line of text shows the reference sequence taken from the GenBank database (X58075.1); below are the sequences of repeats in the human genome obtained from the UCSC Repeat Browser. Dots indicate sites that match the reference sequence; letters indicate positions that do not match. Primer hybridization sites are highlighted in yellow; variable regions are highlighted in green, the presence of which was used to filter reads specific to different LINE-1 subfamilies.

Statistical analysis was performed using the Statistica 10.0 software package (StatSoft, USA). The Mann–Whitney rank test was used to compare methylation levels between groups of samples. To determine the thresholds for outliers in the group of induced abortions, the formulas $Q_1 - 1.5IQR$ and $Q_3 + 1.5IQR$ were used, where Q_1 and Q_3 are first and third quartiles, respectively, and IQR is the interquartile range. Differences were considered significant at $p < 0.05$.

RESULTS

After filtering all reads by LINE-1 subfamilies, it was found L1PA3 subfamiliy was the most represented one (21%), and the majority of reads belonged to the L1HS, L1PA2, and L1PA3 subfamilies (a total of 55 and 54% in the SA and IA groups, respectively) (Figs. 2a, 2b). The remaining subfamilies accounted for a total of less than 1% of all reads. Less than 0.1% of

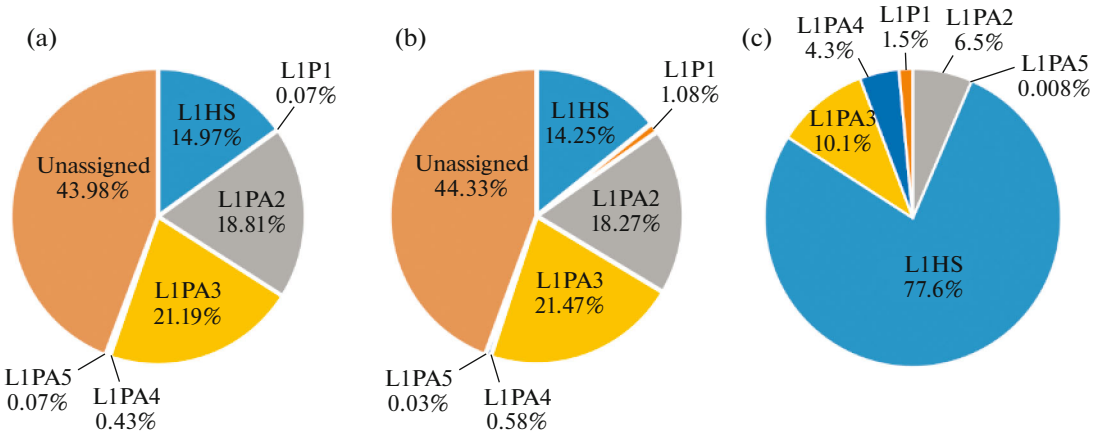

Fig. 2. Distribution of reads between LINE-1 subfamilies (a) in the group of spontaneous abortions (SA) and (b) in the group of induced abortions (IA); (c) representation of LINE-1 subfamilies in the group of unassigned reads.

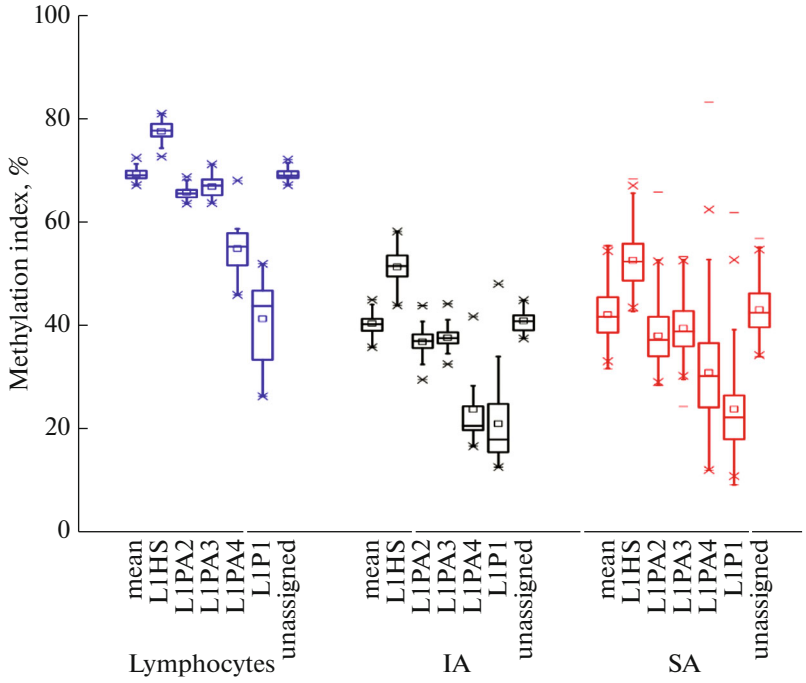

Fig. 3. The average methylation index of the LINE-1 retrotransposon and the methylation index of individual LINE-1 subfamilies in peripheral blood lymphocytes of adult individuals and in the chorionic villi of induced abortions (IA), and a combined group of spontaneous abortions with different karyotypes (SA). Box—25–75% percentiles, whiskers—outlier boundaries; the stripe in the box indicates the median, the square indicates the average value, the crosses indicate the values of 1 and 99%, and the horizontal lines indicate the minimum and maximum values.

reads fell into the L1PA5 subfamily, and therefore it was not used in further analysis. About 44% of reads could not be classified as belonging to one of the LINE-1 subfamilies (Figs. 2a, 2b). Perhaps this is due to genetic variants that arose at individual sites in the regions for which the classification of subfamilies was carried out. Therefore, for unassigned reads, the degree of identity of their sequences to the consensus sequences of various LINE-1 subfamilies was determined. It was found that the majority of all unassigned sequences (77.6%) were closest to the L1HS subfamily (Fig. 2c). Thus, about 48% of all reads belonged to the L1HS subfamily.

The level of methylation in peripheral blood lymphocytes of adults and chorionic villi of induced and spontaneous abortions differed between different subfamilies of LINE-1, being the highest for the evolutionarily youngest subfamily L1HS, specific to humans and retaining copies of LINE-1 capable of retrotransposition (Figs. 3, 4). The level of methyla-

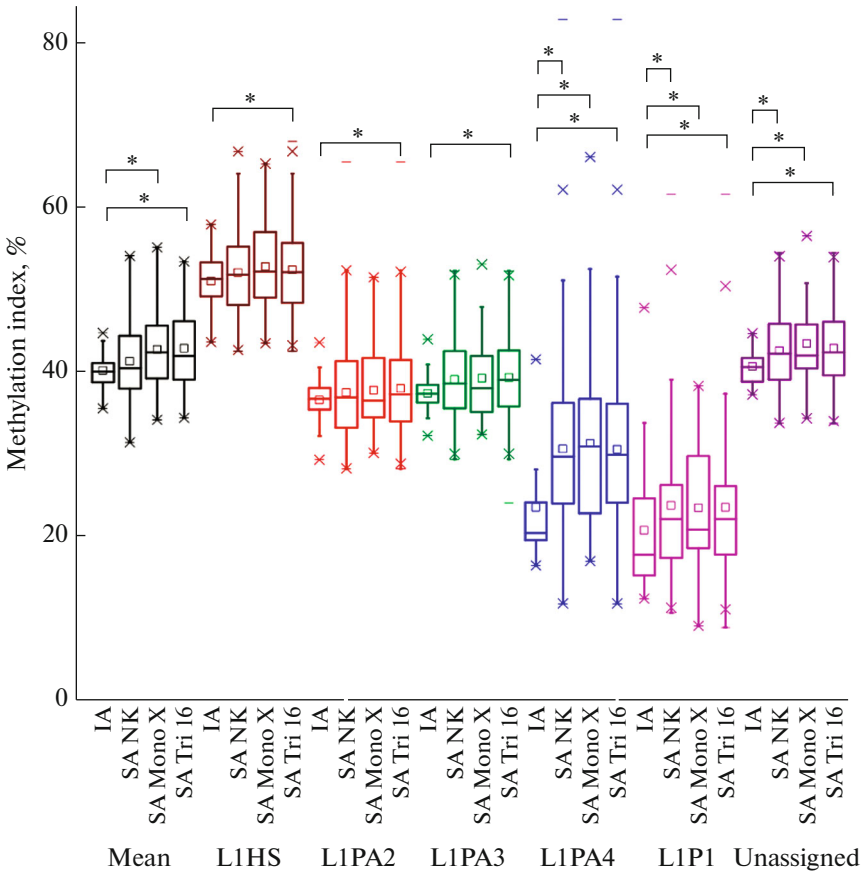

Fig. 4. The average methylation index of the LINE-1 retrotransposon and the methylation index of individual LINE-1 subfamilies in the chorionic villi of induced abortions (IA) and spontaneous abortions with a normal karyotype (SA NK), with monosomy X (SA Mono X), with trisomy 16 (SA Tri 16). Box—25–75% percentiles, whiskers—outlier boundaries; the stripe in the box marks the median, the square marks the average value, the crosses mark the values of 1 and 99%, and the horizontal lines mark the minimum and maximum values; non-distributed—reads not filtered into groups based on the results of the analysis; * $p < 0.05$.

tion was lower for the more evolutionarily ancient subfamilies L1PA2 and L1PA3, and minimal for the most ancient subfamily L1PA4. The exception was the evolutionarily young L1P1 subfamily, for which the methylation level was at the level of more ancient subfamilies (Figs. 3, 4). The identified trend toward a decrease in the level of methylation of various LINE-1 subfamilies from evolutionarily young to more ancient (with the exception of L1P1) in peripheral blood lymphocytes of adults and chorionic villi corresponded to that in tumor samples of various localizations [19]. This indicates a general trend in tissues of different localizations, including tumor cells.

Previously, we discovered an increased level of LINE-1 methylation in spontaneous abortions with an aneuploid karyotype [14]. When analyzed separately in each subfamily, the LINE-1 methylation index did not differ significantly between induced abortions and a pooled sample of all spontaneous abortions for younger subfamilies L1HS (IA—51.1 ± 3.1%; SA—52.6 ± 5.0%, $p = 0.12$) and L1PA2 (IA—36.6 ± 2.8%; SA—37.9 ± 5.4%, $p = 0.3$). The methylation index of older LINE-1 subfamilies was higher in the pooled sample of spontaneous abortions compared to induced abortions: L1PA3 (IA—37.4 ± 2.3%; SA—39.4 ± 5.0%, $p = 0.02$) and L1PA4 (IA—23.5 ± 7.1%; SA—30.8 ± 9.9%, $p < 0.001$). The methylation index in unassigned reads was also higher in spontaneous abortions (IA—40.7 ± 2.0%; SA—43 ± 4.5%; $p = 0.001$). The exception was the evolutionarily young subfamily L1P1 (IA—20.8 ± 8.4%; SA—23.5 ± 8.0%, $p = 0.004$), for which the methylation level was also increased in the group of spontaneous abortions.

When comparing the LINE-1 methylation level of each individual subgroup of spontaneous abortions with induced abortions, the results varied. Spontaneous abortions with trisomy 16 had significantly higher mean LINE-1 methylation levels and methylation levels of all LINE-1 subfamilies compared to induced abortions (Fig. 4). For spontaneous abortions with monosomy X, a significantly higher level of LINE-1 methylation compared to induced abortions was observed for the L1PA4, L1P1, unassigned reads, and average LINE-1 methylation levels. Spontaneous

Table 1. Proportion of spontaneous abortions with a LINE-1 methylation level that goes beyond the boundaries of normal variation in the group of induced abortions

		Average	L1HS	L1PA2	L1PA3	Unassigned reads	Total
Boundaries of the methylation index for IA (nonoutlier range)		33.37–45.73%	39.82–61.32%	29.96–42.52%	32.88–42.73%	35.62–46.76%	
IA	hyper	0	0	1 (2.6)	1 (2.6)	0	39
	hypo	0	0	1 (2.6)	1 (2.6)	0	
SA NK	hyper	34 (19.7)	6 (3.5)	27 (15.6)	41 (23.7)	28 (16.2)	173
	hypo	4 (2.3)	0	7 (4.0)	19 (11.0)	9 (5.2)	
SA Mono X	hyper	11 (23.9)	5 (10.9)	10 (21.7)	10 (21.7)	11 (23.9)	46
	hypo	0	0	0	4 (8.7)	1 (2.2)	
SA Tri 16	hyper	18 (29.0)	2 (3.2)	17 (27.4)	16 (25.8)	15 (24.2)	62
	hypo	0	0	1 (1.6)	2 (3.2)	0	
Total		67 (20.9)	13 (4.1)	64 (20.0)	94 (29.4)	64 (20.0)	320

The number and percentage of abortions with a methylation level higher than the upper threshold (hyper) and lower than the lower threshold (hypo) of the range of variation in the group of induced abortions are presented. IA—induced abortions, SA NK—spontaneous abortions with a normal karyotype, SA Mono X—spontaneous abortions with monosomy X , SA Tri 16—spontaneous abortions with trisomy 16.

abortions with a normal karyotype had increased methylation levels only for the subfamilies L1PA4, L1P1 and unassigned reads (Fig. 4).

In all groups of spontaneous abortions, the coefficient of variation (CV) was twice as high as the variation of LINE-1 methylation (SA NK—11.6%, Mono X—11.4%, Tri 16—10.55%) in the group of induced abortions (5.5%) (Fig. 4). As a result, a significant proportion of spontaneous abortions had a LINE-1 methylation level above the outlier threshold in the group of induced abortions (Table 1). The proportion of spontaneous abortions with the level of LINE-1 methylation beyond the boundaries of normal variation in the group of induced abortions increased with increasing evolutionary age of the LINE-1 subfamily in the series L1HS, L1PA2, L1PA3.

DISCUSSION

Analysis of the methylation levels of various subfamilies of the LINE-1 retrotransposon confirmed our earlier results [14] and showed that, in the chorionic villi of spontaneous abortions, both the average level of methylation of the LINE-1 retrotransposon and the methylation level of individual subfamilies of the LINE-1 retrotransposon are increased. Moreover, for evolutionarily more ancient subfamilies, the increase in the level of LINE-1 methylation relative to the level characteristic of the control group of induced abortions was more pronounced compared to evolutionarily young families. This may be due to the fact that the most evolutionarily young LINE-1 subfamily (L1HS) is still capable of retrotransposition and is under more stringent control owing to DNA methylation in the promoter [19], which is confirmed by the results of the present study.

The role of the LINE-1 retrotransposon in the placenta is unclear. Despite the increased methylation levels found in the present study in the relatively young LINE-1 subfamilies (L1HS, L1PA2, and L1PA3), they are the most transcriptionally active in the placenta [20]. It has been shown that, among the genes capable of using LINE-1 promoters, genes expressed in the brain and placenta are enriched [21]. In addition, retrotransposons from the L1PA2 subfamily may provide their antisense promoters as alternative promoters of long noncoding RNAs in the placenta [22]. It is possible that LINE-1 elements incapable of retrotransposition gradually begin to be used by the genome as separate functional elements (primarily promoters) for nearby genes [23]. In this case, an increase in the level of LINE-1 methylation can lead to disruption of

the expression of adjacent genes, as we have recently shown [24], and disrupt the development of the placenta.

The increased level of methylation was more pronounced for spontaneous abortions with aneuploidy (trisomy 16, monosomy X), but was also observed in a fifth of spontaneous abortions with a normal karyotype. This indicates the possible role of genome methylation disorders as an independent factor, independent of the presence of aneuploidy, associated with embryo death in early pregnancy.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures performed in human research comply with the ethical standards of the institutional and/or national research ethics committee and the 1964 Declaration of Helsinki and its subsequent amendments or comparable ethical standards.

Informed voluntary consent was obtained from each of the participants included in the study.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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