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Origins of Intraindividual Genetic Variation in Human Fetuses

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Abstract

Background: Intraindividual copy number variation (CNV) origin is largely unknown. They might be due to aging and/or common genome instability at the preimplantation stage while contribution of preimplantation in human intraindividual CNVs occurrence is unknown. To address this question, we investigated mosaicism and its origin in the fetuses of natural conception. **Methods:** We studied normal fetuses following therapeutic abortion due to maternal indications. We analyzed the genome of 22 tissues of each fetus by array comparative genomic hybridization for intraindividual CNVs. Each tissue was studied in 2 microarray experiments; the reciprocal aberrations larger than 40 Kb, identified by comparing tissues of each fetus, were subsequently validated using quantitative polymerase chain reaction. **Results:** Through intraindividual comparison, frequency of reciprocal events varied from 2 to 9. According to the distribution pattern of the frequent CNV in derivatives of different germ layers, we found that its origin is early development including preimplantation, whereas CNVs with low frequency have occurred in later stages. Shared CNVs in both fetuses were belonged to thymus and related to the functional role of genes located in these CNVs. **Conclusions:** The origin of some of fetal CNVs is preimplantation stage. Each organ might inherit CNVs with an unpredictable pattern due to the extensive cell mixing/migration in embryonic development.

Keywords

mosaicism, preimplantation, copy number variation, fetus

Background

Intraindividual genetic variation has been reported in diseases with or without a genetic component.[1-4] Such genomic differences are caused by postzygotic events. Although mosaicism may happen in all stages of life, the frequency of detectable clonal mosaicism increases through aging.[4] Mutations during the early postzygotic cell divisions may generate mutated stem cell lineages in the beginning of life, which may increase the risk of cancer and neurodegeneration.[5] Mosaicism is prevalent at the preimplantation stage, ranging from small copy number variations (CNVs) to aneuploidies.[6,7] A single-cell analysis of all blastomeres from good quality human embryos at the cleavage stage showed that embryos were mainly mosaic and only 9% of embryos were diploid in all blastomeres.[6] The frequency of mosaic embryos is reduced at the blastocyst stage but is still common.[8,9] Mosaicism is also highly frequent in miscarriages.[10,11]

Cell differentiation seems to be a barrier for mosaicism elimination[12] by death and/or reduction in abnormal cell division.[7] For instance, checkpoint-dependent apoptosis in aneuploid cells upon differentiation is a mechanism in cultured embryonic stem cells.[13] However, although mosaicism in alterations such as CNVs may be compatible with live birth, the distribution of the mosaic genomic alterations could change penetrance and expressivity.[3] Discordant monozygotic twins for genetic diseases[14] and chromosomal mosaicism in the developing brain[15] are indirect evidences for the early

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embryonic origin of some mosaic genomic alterations; nevertheless, no direct evidence has yet been reported on the origin of these alterations. A postmortem study on several tissues of patients, aged 45 to 85, who died due to diseases without a strong genetic component showed extensive CNVs in different tissues of the same individual.[2] They demonstrated the existence of intraindividual CNVs; however, the time of their occurrence was not clear. These CNVs may have occurred in hotspot regions of genetic variation, leading to common CNVs through separate events during life, or may have risen from events with shared origin during the early embryonic development. Although DNA repair pathways are activated at the preimplantation stage,[16] especially in embryos with DNA damage,[17] this mechanism does not seem to have the ability to eliminate all preimplantation genetic aberrations. Animal studies report high increase of genomic instability in in vitro fertilized embryos compared to in vivo fertilization,[18,19] while such studies are not possible in human. Our study, by comparing the genome of different tissues of normal human fetuses from natural conception, reveals fetal mosaicism of preimplantation and postimplantation origins.

Methods

The scientific considerations was approved by review board of Royan Institute. The study was conducted in compliance with the Helsinki Declaration of the World Medical Association.

Microarray DNA Analysis

The normal fetuses that were obtained after legal therapeutic abortion due to maternal indications were considered as the participants of this study after informed consent was taken from the parents. DNA was extracted from dissections of 25 tissues including somatic, germinal, and extraembryonic tissues as previously described.[20] DNA samples were quantified by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). We analyzed the high-quality samples by array comparative genomic hybridization (aCGH) in a simple loop designed separately for samples of each fetus. This dye-swap approach results in dye balance by labeling every sample with Cy3 and Cy5 dyes alternately. In addition, the loop design, instead of fixed reference, creates the opportunity to maximize exploring variation.[21]

Samples were hybridized to the Oxford Gene Technology (OGT) 180K+15K array (CytoSure Syndrome Plus v2, Leuven, Belgium) according to the manufacturer's instructions (OGT, Oxford, United Kingdom). After hybridization and washing, the slides were analyzed on an Agilent G2565CA microarray scanner with the Feature Extraction Software (Agilent Technologies, Wokingham, United Kingdom). The output text files were imported into CytoSure Interpret Software (OGT), and copy number calling was performed using the circular binary segmentation method. Copy number variations with at least 4 probes were considered for more next analyses. Of these CNVs, only reciprocal CNVs and larger than 40 Kb were included for more validation. It means a high confidence due to calling of the same CNV in 2 separate microarray experiments.

Validation of CNVs by Quantitative Polymerase Chain Reaction

Reciprocal CNVs were identified with high confidence and confirmed by quantitative polymerase chain reaction (qPCR) as an independent method. Those reciprocal CNVs which were not confirmed by qPCR were excluded from data analyses. Primers for qPCR were designed with Primer3plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi). Presence of any repeated sequences was avoided by using Repeat Masker available on the UCSC Genome Browser (http://genome.ucsc.edu/). The qPCR was carried out in duplicate for both target genes and *ZNF80*, as the reference gene, on Applied Biosystems StepOnePlus (Applied Biosystems, CA, USA). Relative copy number was calculated as described.[22] The CNVs were validated by comparing each tissue with its reference tissue according to the simple designed loops (Supplemental Figure 1).

Comparison of All Tissues

For further statistical analysis and pairwise comparison of all tissues, the output text files of all tissues were merged, and pairwise fold changes were calculated. The hierarchical clustering of tissues based on CNVs and the respective heatmap were generated using R.[23]

Comparison With Normal Reference

To validate the genomic CNV observed, samples from the pancreas, colon, gonad, kidney, small intestine, cardiac muscle, and liver of the first fetus were compared with the normal DNA reference by using aCGH.

Data Access

Data sets from this study have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE104314.

Results

The maternal indications for therapeutic abortion of the 2 fetuses were thyroid cancer (fetus 1) and small uterus (fetus 2). Of the 25 tissues collected from fetuses 1 and 2, 21 and 22 tissues had good quality DNA and were hybridized for aCGH analysis. Supplemental Figure 1 shows the simple loops separately designed for aCGH experiments on samples of each fetus.

Frequency of Intraindividual Copy Number Changes Is Variable

Upon the analysis of aCGH data, we detected 185 events in the tissues of fetus 1 which were mapped to 62 genomic regions. Among all events, 67 (36%) were reciprocal and mapped to 13 regions. In fetus 2, 100 events were identified mapping to 58 regions. Among these events, 42 (42%) were reciprocal and mapped to 11 regions.

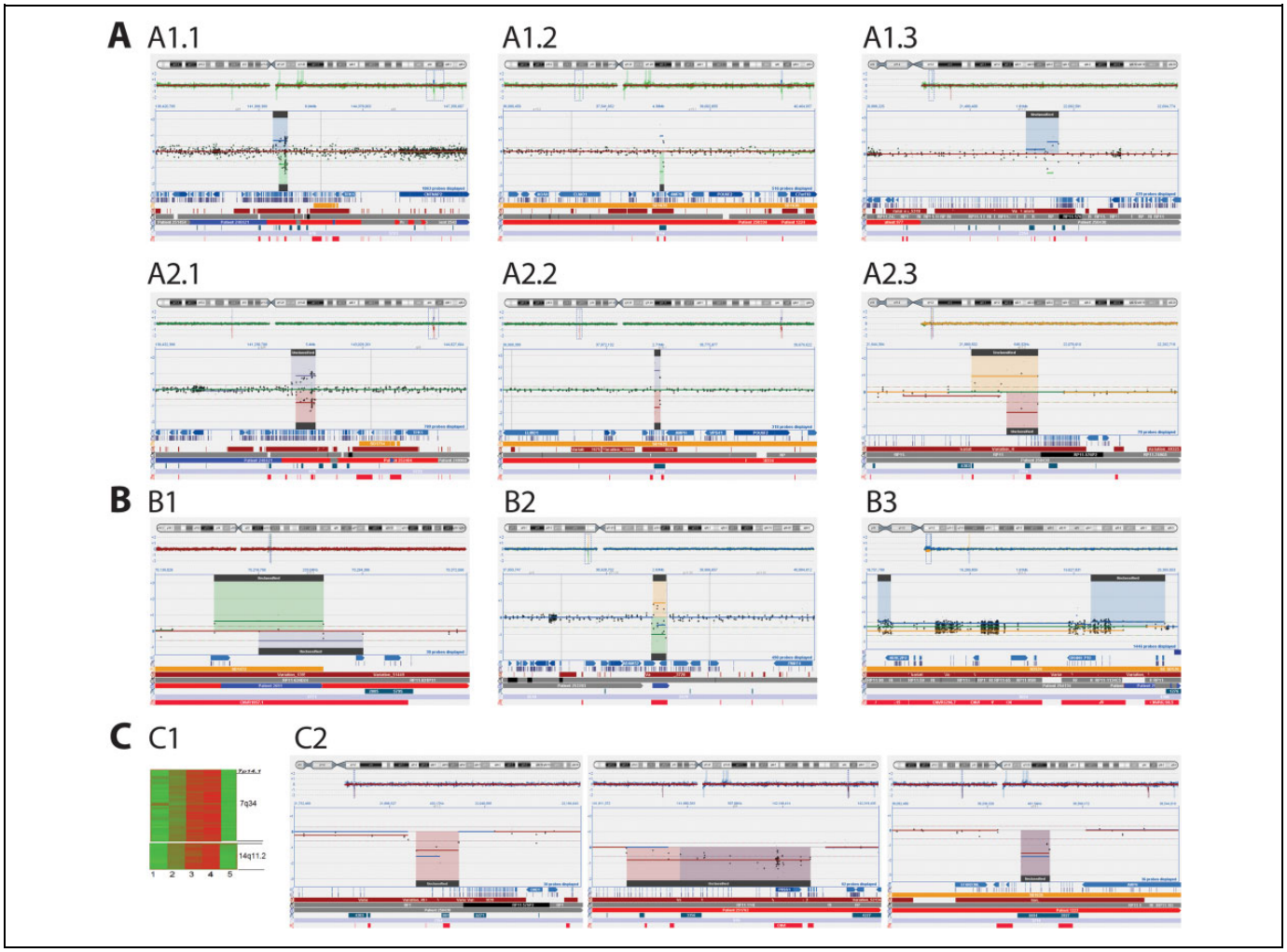

Figure 1. Multiple comparisons of some reciprocal CNVs (A) shared CNVs, A1.1. 7q34 fetus 1 gain: kidney, loss: thymus, baseline (Bl): skin A2.1. 7q34 fetus 2 gain: skin, loss: thymus, Bl: stomach. A1.2. 7p14.1 fetus 1 gain: kidney, loss: thymus, Bl: skin A2.2. 7p14.1 fetus 2 gain: skin, loss: thymus, Bl: stomach A1.3. 14q11.2 fetus 1 gain: kidney, loss: thymus, Bl: spleen A2.3. 14q11.2 fetus 2 gain: skin, loss: thymus, Bl: lung; (B) fetus 2 specific CNVs, B1. 4q13.2 gain: amnion loss: cardiac muscle Bl: chorionic villi B2. 8p11.23 gain: amnion, loss: lung and cardiac muscle. B3. 15q11.1-11.2 gain: cardiac muscle, loss: amnion, Bl: lung. C, Shared deletions in thymus of both fetuses. C1: in the heatmap, red and green colors indicate loss and gain, respectively. Lanes 3 and 4 are thymus tissues of the fetuses 1 and 2. Lane 1 and 5 represent reference tissues against thymuses, cardiac muscle of the fetus 1, and lung of the fetus 2, respectively. C2: shared losses in the thymus of both fetuses and the located genes; left to right, 14q11.2 (*TRDV2*), 7q34 (*PRSS1, PRSS2*), and 7p14.1 (*TRGV9, TRGV11*). CNV, copy number variation.

By comparing the frequency of highly confident CNVs (reciprocal events larger than 40 Kb), 6 of 8 and 23 of 24 events, mapped to 3 and 8 regions, were validated by qPCR in fetuses 1 and 2, respectively (Table 1). Of them, CNV at 8p11.23 of the fetus 2 was explored in 9 experiments, while the others were limited to 2 experiments (Table 2). Supplementary Figure 2 visualizes intraindividual differences using heatmap for the CNV on 8p11.23 of fetus 2.

A Mosaic Pattern for Most of the Called CNVs

Analysis of the CNVs by aCGH and qPCR consistently showed that the quantity of the changes was mostly noninteger multiples, suggesting mosaicism in cells of dissected tissues. This general pattern was visible in many pairwise tissue comparisons (Figure 1).

Similarities and Differences of CNV-Based and Derivative-Based Clustering

In the hierarchical clustering based on initially identified CNVs, some derivatives of the same embryonic origin, for instance, clustering of cerebrum with cerebellum as ectodermal derivatives, and esophagus with small intestine and colon as parts of the digestive tract with endodermal origin are notable in fetus 2 (Supplementary Figure 3A). Nevertheless, there were deviations from the expected clustering patterns of the 3 germ layer derivatives (Supplementary Figure 3B).

Table 1. Frequency of Initially Identified, Highly Confident (Reciprocal and >40 Kb), and Validated CNVs in the Tissues of Both Fetuses.

	CNVs Frequency					
	Fetus 1			Fetus 2		
Tissue/Organ	Initially Identified	Highly Confident	Validated by qPCR	Initially Identified	Highly Confident	Validated by qPCR
Bone	27	0	0	NA	NA	NA
Small intestine	9	1	0	0	0	0
Kidney	11	3	3	4	1	1
Colon	27	0	0	3	1	1
Thymus	25	3	3	2	3	3
Skeletal muscle	6	0	0	1	1	1
Skin	5	0	0	4	3	3
Lung	9	0	0	2	1	1
Amnion	3	1	0	11	5	4
Genital tuberculum	3	0	0	NA	NA	NA
Cardiac muscle	11	0	0	18	3	3
Esophagus	2	0	0	1	0	0
Spleen	2	0	0	2	0	0
Pancreas	12	0	0	4	0	0
Liver	6	0	0	1	0	0
Aorta	4	0	0	NA	NA	NA
Cartilage	8	0	0	7	1	1
Cerebrum	1	0	0	5	0	0
Stomach	1	0	0	4	1	1
Cerebellum	3	0	0	4	1	1
Gonad	10	0	0	7	2	2
Cord blood	NA	NA	NA	0	0	0
Chorionic villi	NA	NA	NA	8	1	1
Cornea	NA	NA	NA	12	0	0
Nail	NA	NA	NA	0	0	0
Total	185	8	6	100	24	23

Abbreviations: CNV, copy number variation; NA, not applicable; qPCR, quantitative polymerase chain reaction.

Table 2. Highly Confident CNVs (Reciprocal Events Larger Than 40 Kb) in Both Fetuses Confirmed by qPCR.[a]

Fetus No.	CNV No.	Location	Start Range	End Range	Size Range (bp)	Genes	Event Frequency
1	1	7P14.1	38290457	38336041	45584	*TRGV11, TRGV9*	2
	2	7q34	141801737 to 141967536	142197140	229604 to 395403	*PRSS2, TRBV5-4, TRB, PRSS1*	2
	3	14q11.2	21814127 to 21936543	21972884 to 22000569	36341 to 186442	*TRDV2*	2
2	1	4q13.2	70183174 to 70216706	70264747 to 70294386	77680 to 81573	*UGT2B28*	2
	2	7P14.1	38290457	38336041	45584	*TRGV11, TRGV9*	2
	3	7q34	141791795 to 141872841	142197140	324299 to 405345	*PRSS2, TRBV5-4, TRB, PRSS1*	2
	4	8p11.23	39368506 to 39353287	39482072	78303 to 128785	*ADAM5P*	9
	5	11q11	55136664 to 55142200	55207471	65271 to 70807	*OR4P4, OR4C6, OR4S2*	2
	6	14q11.2	21863210	22000569	137359	*TRDV2*	2
	7	15q11.1-11.2	18261165 to 18810004	19534662 to 19534662	62876 to 1273497	*U6, VSIG7, HERC2P2*	2
	8	15q11.2	19871277 to 19916779	20082731 to 20297653	211454 to 380874	*RPS8P10, VSIG6, SRP euk arch, hsa-mir-1268, OR4N4*	2

Abbreviations: CNV, copy number variation; qPCR, quantitative polymerase chain reaction.
[a]Last column shows frequency of the events in analyzed tissues. The mentioned locations are based on *build hg18* of the human genome UCSC genome browser.

Shared CNVs in Both Fetuses

By comparing the validated CNVs between the 2 fetuses, 3 CNVs were present in both fetuses, all related to thymus.

Discussion

We selected fetuses of the same gestational week to minimize the likely effect of developmental stage on genomic variation. Comparison of the genome of different tissues of the same fetus revealed that different tissues could have genomic differences at the CNV level. One of CNVs was detected in several tissues of the fetus 2 (on 8p11.23); this is because events occurring in a more primitive derivation will appear in more tissues, while CNVs with lower frequency are likely to have occurred in the later stages of embryonic development. Observation of CNVs in several organs, particularly those present in derivatives with different origins, indicates that they originate at the early stages of embryonic development, preimplantation, or postimplantation.

Copy number variations might have, nevertheless, occurred in a portion of biopsied cells. Changes less than one copy number shows that all of the biopsied cells have not undergone the same change which is either due to the different origins of biopsied cells and/or occurrence after tissue differentiation.

Although the diagnosis of genetic diseases is often based on blood sample examination, mutations limited to one type of tissue other than blood could lead to tissue-specific diseases such as schizophrenia, Alzheimer, autism, and heart disease.[3] According to the results of somatic mosaicism studies, the genomic status of a tissue at a time is not an indicator for all tissues of person for a whole lifetime.[24] Mosaicism usually causes development of a milder form of the disease when compared with total abnormality. The other issue is that analysis of available samples in prenatal diagnosis (PND) such as amniotic fluid cells and chorionic villi is not necessarily good indicators of the fetal genomic status, similar to our observation for the amniotic sample of fetus 2 (Supplementary Table 2). Regarding the evidence for genomic differences of different tissues in the same person, it seems biopsy of the affected tissue is vital in evaluating the genomic contribution. Analysis of developmentally unrelated tissues might falsely rule in events that are unrelated to disease and its maintenance.[2]

Recent reports indicate that transfer of euploid–aneuploid mosaic mouse[25] and human[26,27] embryos with mosaic whole chromosomal and/or segmental aneuploidy[27,28] have led to live birth. Human embryos containing 30% to 50% abnormal cells led to 33% healthy births,[26] while transfer of mosaic mice containing 50% and 25% abnormal cells led to 44% and 100% live births, respectively.[25] However, it must be considered that live birth does not equate with normal genomic status in all tissues.

The inconsistencies between tissue clustering and embryonic layer derivative clustering can be attributed to several factors. First, many organs have multiple origins, while each of the origins may harbor different CNVs. Secondly, parenchymal cells are mostly considered in determining the origin, while some sectors, such as blood vessels and nerves present in all collected samples, may have origins other than the parenchyma. Thirdly, another important factor may be the extensive cell migration and cell mixing after formation of the 3 initial layers following gastrulation until the whole process of organ development, resulting in a mosaic organ containing several cell populations from different parts of the inner cell mass.

Table 3. Shared CNVs in the Identical Tissues of Both Fetuses and Genes Located in Each of CNVs.[a]

Tissue/Organ	Chr	Cytogenetic Location	Genes
Thymus	7	p14.1	TRGV9, TRGV11
	7	q34	PRSS1, PRSS2
	14	q11.2	TRDV2

Abbreviation: CNV, copy number variation.
[a]Among 25 studied tissues, the validated shared CNVs were only limited to thymus.

Shared CNVs in the same organ of both fetuses may be related to the functional role of genes located in such CNVs. In this context, genes encoding *TRDV2*, *TRGV9*, *TRGV11*, *PRSS1*, and PRSS2 are notable for copy number changes in both thymus samples (Table 3 and Figure 1C show the thymus deletions in 7p14.1, 7q34, and 14q11.2 regions which including probes for these 5 genes). Deletions in 7p and 14q are in agreement with a recent report[29] that highlights CNV occurrence in these regions of thymus genome due to T-cell receptor gene rearrangements. Finding of such a previously reported CNVs in thymus confirms that these shared CNVs should not be random, while we studied 25 tissues of each fetus and the validated shared CNVs were only limited to thymus.

One of the functional effects of such genetic variation is change in gene expression because CNVs may alter gene dosage by copy number changes.[30] To the best of our knowledge, this is the first direct confirmation of intraindividual genetic variation in the form of CNV in normal human fetuses based on the analysis of a variety of tissues. We increased the number of studied tissues to 25 to have a more complete view from the distribution of CNVs at this level. Study of pure embryonic derivative microdissections and/or single-cell CNV profiling using SNP array and/or next-generation sequencing seems to result in clustering reconstruction and may address other pertinent questions such as each CNV occurrence timing.

Although the revealed CNVs had mainly limited distribution, since the CNV located on 8p23.1 of the fetus 2 distributed in different germ layers, it should be of a preimplantation origin. A recent study highlighted the role of perizygotic development in CNV mutation by analyzing blood samples of patients with developmental disorders carrying multiple de novo CNVs.[31] They concluded that a CNV mutator is turned on only in early development, while pluripotency minimizes their effect on development. This study did not rule out CNV occurrence in gametogenesis, while parent of origin for the reported de novo CNVs was mainly maternal. A hypothesis

is that preimplantation genome inactivation increases the susceptibility of CNV mutation in the early embryonic cell divisions through decrease in zygotic storages.[32] By embryonic genome activation in the 4- to 8-cell stage and increase in gene expression, the mutator mechanism gets turned off. The identified CNVs in our study should be de novo since we compared different tissues of the same fetus in a pairwise manner. Although we did not examine the parents, we believe that inherited CNVs and those with a gametogenesis origin have been excluded in our study, since all CNVs were identified by comparing a sample with a reference from the same fetus, revealing their postzygotic origin. The frequent CNV observed here with a preimplantation origin may have risen as a result of the activation of preimplantation CNV mutators. We did not observe any pathogenic CNV in the studied tissues of the fetuses; however, it should be considered that somatic variation could have pathological consequences.[33] In such CNVs, those with preimplantation origin increase the risk of complication. We suggest that despite live births could occur by transfer of mosaic embryos, since it could significantly increase the risk of early/late onset diseases with a genetic component in future babies, this approach must be further evaluated before its routine integration into Preimplantation Genetic Screening (PGS) as suggested by Greco et al. Blastocyst biopsy, due to the availability of several cells[7,9] in combination with genomic testing methods able to detect segmental pathogenic aneuploidies, seems to be the optimal approach for mosaicism diagnosis and selection of healthier embryos in PGS for cases with a family history or products of conception. PGS methods which are able to detect mosaicism and CNVs are recommended for the transfer of healthier embryos, especially for families with a risk of segmental aneuploidies.

Conclusions

The intraindividual genomic differences in the studied fetuses seem to occur at the early stages of life. Given the distribution of CNVs in multiple fetal tissues, including derivatives of different embryonic layers, origin of such CNVs is likely to be at the preimplantation stage. Like other studies of mosaicism, we note that results of one tissue, such as blood, in routine clinical diagnosis or chorionic villi/amnion in PND are not necessarily good indicators of the whole body. Shared CNVs in the same organ of both fetuses like what we observed in the thymus may be related to the functional role of genes located in such CNVs.

Authors' Note

M.B., H.G., B.M., and M.R.V. contributed to the conception and design of the work and interpretation of data. M.B. performed microarray experiments, analysis of data, drafting the work, and revising it critically for important intellectual content. A.K-.F. performed comprehensive analysis of microarray data. P.B.B. performed qPCR experiments, and K.A. performed DNA extraction from a variety of tissues. H.G. provided the fund and supervised the study. All coauthors approved the final version to be published and agreed to be accountable for all aspects of the work. This study was approved by Research Ethics Committee of Royan Institute, Reference Number: EC92/1003.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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