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ORIGINAL RESEARCH

Loss of Imprinting of *IGF2* Gene in the Chorionic Tissues of Spontaneously Eliminated Human Embryos

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Abstract: Insulin-like growth factor-2 (IGF-2) is a mitogen, growth and differentiation modulator for many cell types. It is mainly expressed during the prenatal development, and its activity strongly depends on the genomic imprinting. Genomic imprinting in the chorionic tissues of spontaneously eliminated human embryos has been studied on the model of 820-AG (Apa1) of the *IGF-2* gene locus. Molecular and genetic analysis was performed on the polymorphic *820-AG IGF2* locus in 107 samples of DNA extracted from the chorionic tissues of spontaneously eliminated human embryos within 5–10 weeks of gestation. Presence of AG genotype Apa1 single nucleotide polymorphisms of the IGF-2 was shown to cause more than a 7-fold increase in the risk of embryo elimination. Thus, the loss of genomic imprinting of the *IGF-2* gene may be an important cause of the miscarriages in human.

Keywords: IGF-2, loss of imprinting, human spontaneous eliminated embryos

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Introduction

There is a group of diseases with an atypical mode of inheritance resulting from a phenomenon wherein genomic imprinting is based on unusual expression of the homologous genes received by the progeny from parents. The "imprinting" term was first used in genetics by G. Crouse for explaining the elimination of parentally transmitted chromosomes in insects.[1] A majority of studied genes are known to have the bi-allelic expression. However, there is also a range of genes localized in so-called imprinted regions that are characterized by the monoallelic expression when only the paternal or maternal allele is expressed, while the other one is inactive at expression.[2,3] Random suppression of genes occurs during their transmission by the female or male generative cells. Thus, genomic imprinting is an epigenetic process leading to stable functional divergence of the homologous genes received from one of parents. It was shown that random methylation of DNA during spermatogenesis or oogenesis, which results in ceasing a transcription of genes of the imprinted region, plays a leading role in the genesis of genomic imprinting.[4]

A number of paternal and maternal genes are known to indirectly affect fetus weight, placenta development, and other characteristics of prenatal development. The location of the imprinted genes is definitely fixed to human chromosomes 7, 11 and 15. These genes are also supposed to be present in chromosomes 2, 3, 6, 14 and 20. Not less than 60 imprinted genes, frequently grouped in clusters, have been identified.[5,6]

IGF-2 regulates the speed of uteral, placental and fetal growth. Aside from this, *IGF-2* stimulates trophoblast growth, placenta expansion and maturation, and its dysfunction may cause miscarriage. Analysis of single nucleotide polymorphisms (SNP) in the *IGF-2* gene has been proposed as a prognostic marker of the risk of muscle disorders, malfunction of prenatal development, and carcinogenesis.[7–9]

Recurrent spontaneous abortions (RSA) are spontaneous eliminations of the fetus with the same biological partner before the 24th week of pregnancy, and they are observed in 0.5%–3% of married couples. RSA has a broad factorial etiology including both the non-genetic factors (hormonal, anatomic, immune, infectious, hemostatic, metabolic, environmental, as well as drugs, stress and tumors) and genetic factors (quantitative and structural chromosome abnormalities and some monogenic pathologies).[10]

Women with pregnancy losses in their anamnesis have higher risk of further abortions, which implies that some idiopathic RSAs can be of genetic etiology. Changes in specific genes that potentially influence RSA, include mutations affecting physiological mechanisms in the organism and causing variations in the activity of maternal or paternal genomes that are functionally non-equivalent. This functional divergence is based on genomic imprinting and epigenetic changes leading to the monoallelic expression or differential expression of certain chromosome loci and depending on the parental origin of alleles.[10] To summarize, genomic imprinting has been shown to play an important role in development of the placenta and embryo in humans.

It has been detected that the 820 G/A polymorphism of the *IGF-2* gene and the loss of imprinting (LOI) can cause spontaneous abortions.[11] In our study, we present the results of genetic analysis of 107 samples of DNA extracted from the chorionic tissues of spontaneously eliminated human embryos obtained within 5–10 weeks of gestation. In 90% of examined cases, the bi-allelic expression of the *IGF-2* gene was spotted, which indicates a loss of the imprint status of this genetic locus.

Materials and Methods

Cells of the chorionic villi (n = 107) were obtained via dissection of biological material of spontaneous abortions. They were obtained for genetic studies at the Institute of Hereditary Pathology of Academy of Medical Sciences of Ukraine from the gynecological departments of different Lviv clinics (Regional Hospital, Perinatal Center, and Hospital Ambulance). The gestational age of spontaneous abortions was within 5–10 weeks. As controls, healthy individuals (n = 40) without any burdened obstetric and genetic history were used. Samples of biological material were taken for research after the informed consent of patients.

Isolation and purification of DNA from the chorionic villi cells were performed using enzymatic cleavage of tissue with subsequent phenol extraction. Amplification of DNA sequences was performed by polymerase chain reaction (PCR) in the automatic mode in thermal cycler "Tertsyk" ("DNA Technology", Russian Federation). Restriction endonuclease, thermostable

Taq-polymerase and oligonucleotide primers ("Fermentas", Vilnius, Lithuania) were used as described by Tadokoro et al.[8] Electrophoretic separation of products of PCR-RFLP analysis (Restriction Fragments Length Polymorphism) was conducted in 2% agarose gels that were further stained with the ethidium bromide and scanned on UV trans-illuminator.[3] The 292 bp fragment was amplified by the PCR and treated with *Apa1* endonuclease (37 °C, 16 hours [h]). If the restriction site was absent, the amplified product was not changed and provided the 292 bp fragment (820AA genotype). In cases where the 820GG *IGF-2* genotype appeared in the DNA sample, the 229 bp and 63 bp fragments were observed. In cases where the heterozygous 820AG genotype was present, three fragments of 292, 229 and 63 bp appeared on the electropherogram (Fig. 1).

Isolation and purification of RNA of the chorionic villi cells were performed using Diatom™ RNA Prep 100 set ("Neogen", Russian Federation). For elimination of DNA impurities, the initial solution was treated with DNAase ("Fermentas", Vilnius, Lithuanian Republic). For the high-performance PCR, GenPak® PCR Core set ("Laboratory Isogene", Russian Federation) was used. PCR was performed in the automatic mode by thermal cycler "Tertsyk" ("DNA Technology", Russian Federation). Restriction endonuclease and oligonucleotide primers were from "Fermentas" (Vilnius, Lithuanian Republic).

RNA was reverse-transcribed by 200 units of the reverse transcriptase of Moloney murine leukemia virus with 200 ng of random hexamer, 0.5 mM dNTP, 40 units of RNase inhibitor, 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT. Reverse transcription was performed using GenPak® RT Core set ("Laboratory Isogene", Russian Federation) in a total volume of 20 μL for 1 h at 37 °C. In the control condition, the reaction was performed using DNA-free total RNA in the absence of reverse transcriptase. RT was inactivated by heating at 70 °C for 10 min.

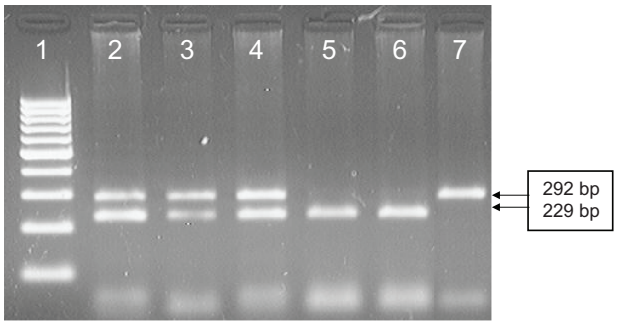

Figure 1. Electrophoresis in 2% agarose gel of *Apa1* restriction fragments of exon 9 of the *IGF-2* gene: 1–100 bp DNA Ladder; 2, 3, 4-820AG genotype; 5, 6-820GG genotype; 7-820AA genotype.

cDNA (50 ng) was amplified by the PCR using primers for the *IGF-2 Apa1* site under the same conditions as used for genomic DNA, except the number of cycles was increased to 40. The amplified cDNA was also digested with *Apa1* (yielding a 292 bp fragment or 292 bp and 229 bp fragments). The presence of 292, 229 and 63 bp fragments in the embryo sample on 2.5% agarose gel indicates the bi-allelic expression. The presence of either 292 bp band or 229 bp and 63 bp bands was considered to indicate the retention of imprinting.

Results

The results of study of the distribution of genotypes of 820G→A *IGF-2* locus in the chorionic villi cells obtained from spontaneous abortions (n = 107) at early stages of human ontogenesis are presented in Table 1. These results were compared with the results of control group consisting of 40 healthy individuals.

Principally different characters of the distribution of genotypes of the *Apa1-IGF2* locus was observed in samples of the spontaneous abortions compared to control samples. As one can see (Table 1), the ratio of GG-, GA- and AA-genotypes in the percentage equivalent is as following: 20.6:69.2:10.2. Statistical analysis including calculation of Pearson coefficient χ^2 showed a statistically significant decrease in the frequency of the GG-genotype and an increase in the frequency of the GA-genotype compared to controls. Thus, the AG-genotype can be considered to be a genotype aggressor, at least in the studied group, while GG-genotype may act as a genotype protector in this group.

Calculation of the odds ratio (OR) at a 95% confidence interval in the case of AG-genotype showed more than 7-times increased probability of the appearance of spontaneous abortions (Table 2).

At the next stage of our study, we have used the Apa1 locus as a model for monitoring the loss of imprinting of the *IGF-2* gene. Such an approach allows for the distinguishing of RNA transcripts only in the presence of the 820-AG Apa1 polymorphism of the *IGF-2* gene. Samples with previously confirmed 820GA genotypes of the *IGF2* gene were selected.

Table 1. The distribution of genotypes of 820G→A *IGF-2* locus in the chorionic villi cells of human spontaneous abortions.

IGF-2 genotype	Control group, n = 40			Studied group, n = 107			χ^2	*P*
	n	%	HWE (*P* = 0.23)	n	%	HWE (*P* = 0.002)		
GG	27	0.675	0.620	22	0.206	0.304	28.87	<0.001
AG	9	0.225	0.335	74	0.692	0.495	25.17	<0.001
AA	4	0.100	0.045	11	0.103	0.201	0.00249	>0.05

After RNA isolation and purification, the samples were subjected to reverse transcription and used as a matrix for PCR. PCR with RNA samples not subjected to reverse transcription was used as control. Total and un-degraded RNA was obtained in 41 cases, and was used for analysis. The nativity of isolated RNA was tested by electrophoresis, and 2 µl of each sample were applied. Electrophoresis of good quality total RNA showed two separate bands corresponding to 28S and 18S RNA (Fig. 2).

It is known that in good quality RNA, the 28S RNA band on the agarose gel should be approximately three times more intensive compared to the 18S band (Fig. 2). Slight differences in intensity of these bands indicates RNA degradation. DNA products obtained after the RT and subsequent PCR were analyzed by restriction analysis, as in the study of genomic DNA. Since the *IGF-2* gene is imprinted, RNA transcripts of only one allele should be present normally in tissues. The detection of two alleles of the 820GA *IGF-2* (heterozygote) can be interpreted as a loss of imprinting which might lead to changes in the expression of the corresponding gene.

As shown in Table 3, in approximately 90% of analyzed cases, samples of the chorionic villi cells were characterized by a loss of imprinting of the *IGF2* gene in the AG-genotype. There were only 9.8% cases found with AA- or GG-genotypes, indicating the imprinting of the *IGF-2* gene.

Discussion

It has been declared that a specific polymorphism of the *IGF-2* gene and the loss of imprinting (LOI) can lead to spontaneous abortions.[12] DNA extracted from the chorionic tissues of spontaneously eliminated human embryos obtained at early stages of gestation is a proper biological material for conducting such analysis. In this study, we used 107 samples of DNA that was isolated from the human embryos of 5–10 weeks of gestation. A significant difference was detected between control and experimental samples in frequency of GG- and AG-genotypes of 820-AG Apa1 polymorphism of the *IGF-2* gene (Table 1). The product of this gene—IGF-2—has been shown to be important in physiological processes of regulation of cellular growth in uterus, placenta and fetus,[6,12] as well as pathological processes leading to the miscarriages in humans.[13] That was one the main reasons why SNP in the *IGF-2* gene could be used as a prognostic marker of the risk of malfunction in prenatal development.[7–9]

We found that the frequency index of the AA-genotype of the 820G→A polymorphic locus of the *IGF-2* gene in spontaneous abortions did not differ from that in the control group of healthy individuals.

Table 2. Odds ratio (OR) index of AG-genotype of insulin-like growth factor-2 gene Apa1 locus in the chorionic villi cells of 5–10-week human spontaneous abortions.

OR (CI 95%)	χ^2	*P*
7.7239 (3.3079–18.035)	25.78	<0.01

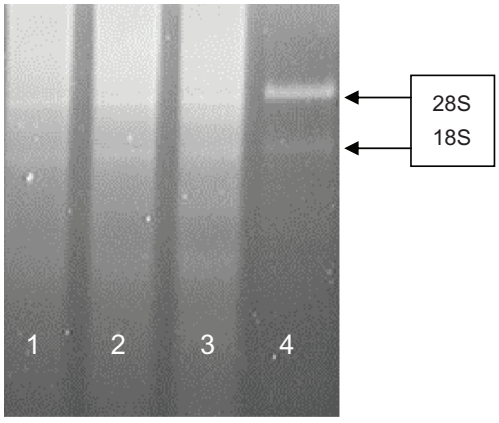

Figure 2. Electrophoresis in agarose gel of total cellular RNA visualized under UV light after the ethidium bromide staining.
Notes: All samples (lines 1–4) show the presence of two bands corresponding to 28S and 16S rRNA. Line 4—good quality RNA with 28S RNA band that is more intensive compared to the 18S band.

Table 3. Analysis of 820-AG Apa1 polymorphism of the *IGF-2* gene in cDNA and of the imprinting status of the *IGF2* gene.

Genotype	n	%
AA*	1	2.4
GG*	3	7.4
AG**	37	90.2

Notes: *AA/GG—normal imprinting; **AG—loss of imprinting.

Since spontaneous abortions were characterized by a significantly increased incidence of AG-genotype and a significantly reduced frequency of GG-genotype compared to the control group, there is a definite reason to consider AG-genotype as a genotype aggressor and GG-genotype as a genotype protector for this group. We found a significantly higher frequency of A-allele in the group with spontaneous abortions compared to the control group. According to a Hardy-Weinberg equilibrium analysis, the distribution of genotypes of 820G→A polymorphic loci in the *IGF2* gene in spontaneous abortions demonstrated that the frequency of the heterozygous GA-genotype was higher than the theoretically expected one. The actual frequency of that genotype was 0.692, while the theoretically expected frequency was 0.495 (pHWE = 0.002).

Obviously, human embryos with such AG genotypes undergo elimination more frequently than the embryos with homozygous AA- or GG-genotypes of the polymorphic locus *Apa1* of the *IGF2* gene. The calculated OR confirmed the correlation between the presence of AG-genotype in the embryo and a significant increase (almost 8 times: OR = 7.7239, CI 3.3079–18.035, $P < 0.01$) in the risk of spontaneous abortion. These results might explain the lack of heterozygotes in control samples.

Lighten et al[12] studied the imprinting process on the mRNA *Apa1-IGF2* model. They found that the genomic imprinting is turned on in the 8-cell human embryo, while in the pre-implantation period functioning of the "parental" allele is blocked. Thus, one can assume that in spontaneous abortions the imprinting mechanism did not start functioning at all, resulting in the termination of development of the organism. Our data also agree with the results of Shen et al[13] who explained the loss of imprinting in conjunction with chromosomal abnormalities in spontaneous abortions. Taking into account that chromosomal abnormalities of human embryos dominate among other causes of early reproductive losses,[13] we believe that a significant increase in frequency of certain genotypes of the *IGF2* gene, which we observed in the spontaneous abortion group, might also be associated with chromosomal abnormalities in the examined embryos. Besides, it looks very probable that the imprinting is necessary for normal development in human ontogenesis, and the loss of imprinting results in its termination at the earliest stages of fetus development.

Since the IGF-2 is of unquestionable importance for normal growth and development of human body and at later stages of ontogenesis,[4–6,8,10,12] auto- and paracrine functions of this growth factor might be tightly dependent upon the appearance of SNP in different regions of the *IGF2* gene. Our findings undoubtedly confirm a significance of polymorphism in the Apa1 region of this gene. Although such a polymorphism does not directly affect changes in the amino-acid sequence of the coded protein, in a combination with other mutations it might cause more significant pathological effects.

Considering the results obtained in our study and data published by other researchers, we believe that single nucleotide 820G→A polymorphism of the *IGF2* gene is functionally significant and contributes to violation of growth and development of human embryos and fetuses.

Conclusions

1. The loss of imprinting of the *IGF2* gene was analyzed in 41 samples of the chorionic villi cells in human. In 90% of cases, the loss of imprinting was detected.
2. The appearance of SNP in the AG-genotype *Apa1* of the insulin-like growth factor-2 gene correlates with more than a 7-fold increase in the risk of embryo elimination (OR = 7.7239).

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Author Contributions

Conceived and designed the experiments: DZ, HM. Analyzed the data: BT. Wrote the first draft of the manuscript: BT, OB. Contributed to the writing of the manuscript: MT. Agree with manuscript results and conclusions: OB, MT. Jointly developed the structure and arguments for the paper: DZ, HM, BT. Made critical revisions and approved final version: DZ. All authors reviewed and approved of the final manuscript.

Disclosures and Ethics

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