Genetic Predisposition to Idiopathic Recurrent Spontaneous Abortion: Contribution of Genetic Variations in IGF-2 and H19 Imprinted Genes

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Problem
Recurrent spontaneous abortion (RSA) is a common clinical problem with a complex etiology of genetic and non-genetic causes, which remains to be fully determined. IGF-2 stimulates trophoblast invasion, proliferation and maturation of placenta, while H19 RNA suppresses growth. As genomic imprinting plays a critical role in the development of placenta and embryo, our aim was to evaluate the possible role of variations in IGF-2 and H19 imprinted genes as factors of predisposition for RSA.

Method of study
A case–control study was conducted to determine the association between IGF-2 and H19 gene polymorphisms and the susceptibility to RSA in 113 couples with RSA and 226 controls. PCR/RFLP were performed to analyze IGF-2 ApaI and H19 HhaI polymorphisms.

Results
We found a statistically significant difference in the genotype frequency distribution of IGF-2 ApaI polymorphism between males from couples with RSA and healthy males ($\chi^2(2) = 45.12$; $P < 0.0001$). There were no differences in the genotype and allele distribution of H19 polymorphism frequencies, or for the IGF-2 ApaI polymorphism between female groups.

Conclusion
The presence of IGF-2 ApaI polymorphism in partners of RSA women could affect IGF-2 level of expression in placenta and embryo and represent a risk factor for RSA susceptibility.

Introduction

Recurrent spontaneous abortion (RSA) is defined as the spontaneous loss of three or more consecutive pregnancies with the same biological partner before the 24th week of pregnancy and it affects 0.5–3% of couples.[1] As the cause of RSA can be determined in only about 50% of cases, it represents an important clinical problem. RSA has a multifactorial etiology that includes non-genetic (hormonal, anatomical, immunologic, infectious, haemostatic, metabolic, and environmental factors, drugs, stress, tumors) and genetic factors (numerical and structural chromosome abnormalities, certain monogenic disorders and gene

variability). In the remaining 50% of cases, the cause remains unknown and thus it is referred to as idiopathic RSA. The fact that women with a history of pregnancy loss have a higher risk for a subsequent miscarriage[2–4] suggests that a part of idiopathic RSA might have a genetic etiology. Until this date, little is known about gene mechanisms and the contributions of zygotic, maternal and paternal genomes to the regulation of functions of the feto-maternal interface.[5] Changes in potentially important genes, which could influence RSA, include mutations that affect physiological mechanisms in a qualitative manner and genetic variability that affects the same mechanisms in a quantitative manner.

Successful pregnancy depends on the presence of both maternal and paternal genomes, which are functionally non-equivalent.[6–8] This functional difference is known as genomic imprinting and is the consequence of epigenetic modifications, which lead to monoallelic expression or differential expression of certain chromosome regions dependent upon parental origin of inheritance. Genomic imprinting plays a critical role in the development of placenta and embryo as evidenced by aberrations of human pregnancy including abnormalities of fetal growth (Beckwith–Wiedemann syndrome, Silver–Russell syndrome etc) and gestational trophoblastic diseases (hydatidiform mole, choriocarcinoma etc).[9,10] Genes undergoing the phenomenon of genomic imprinting are involved in the regulation of major functions at the feto-maternal interface, such as nutrient transport,[11] trophoblast proliferation, invasion[12–14] and angiogenesis.[15,16] So far, all imprinted genes examined for placental expression in mice have been found to be expressed, implicating that they are essential for placental function.[11] As they are evolutionarily conserved in human and expressed in similar extraembryonic tissues, it makes them an important factor, which might explain human pregnancy complications of unknown genetic etiology, including idiopathic RSA.[17] Additionally, it has been found that assisted reproductive technologies in human, especially intracytoplasmic sperm injection (ICSI), are associated with an increased risk of imprinting disorders and other epigenetic processes that control implantation, organogenesis and fetal growth.[18–20] Moreover, RSA may occur because of familial hydatidiform mole syndrome with recurrent hydatidiform moles, which is a consequence of dysregulation of imprinting.[21]

As most recurrent miscarriages occur within the first 12 weeks of conception, we hypothesized that 'imprinted genes' involved in the regulation of early implantation and trophoblast growth could influence the risk of RSA.

Insulin-like growth factor-2 (IGF-2) and H19 are a part of a cluster of imprinted genes on human chromosome 11p15.5.[22] These genes show a coordinate regulation and are reciprocally imprinted, IGF-2 being expressed only from paternal[23] and H19 only from maternal allele.[24] IGF-2 is the major fetal growth factor which regulates feto-placental growth by stimulating trophoblast migration and invasion.[25–28] It has been shown that IGF-2 is a potent mitogen and has an autocrine effect on preimplantation development.[29,30] IGF-2 also regulates diffusional exchange characteristics of the placenta, and mutations in IGF-2 gene lead to intrauterine growth restriction.[31] In human preimplantation embryos IGF-2 and H19 are amongst the first genes to be expressed. Biallelic expression of IGF-2 is already observed in four-cell-stage embryos, while monoallelic expression from the paternal allele is first observed in the eight-cell-stage embryo.[32] The highest levels of IGF-2 and H19 expression are seen in the most proliferative cells of the early human placenta: extravillous trophoblast, fetal endothelial cells, chorion mesenchymal cells and cytotrophoblast where IGF-2 induces cell cycle and modulates trophoblast proliferation and maturation.[33,34]

H19 encodes a functional non-coding RNA which suppresses growth.[35] H19 RNA is expressed during human prenatal period and is normally silenced in most tissues after birth.[36] It regulates the expression of IGF-2 on a transcriptional level and also participates in the regulation of IGF-2 mRNA translation.[37]

In this study, we hypothesized that polymorphisms in IGF-2 and H19 genes represent the risk for RSA susceptibility. For this purpose, we analyzed IGF-2 ApaI and H19 HhaI gene polymorphisms in a group of 113 Slovenian couples with RSA and 113 healthy male and female controls without any previous record of pregnancy loss.

Methods

Subjects

A case–control study was conducted to determine the association between the IGF-2 and H19 gene polymorphisms and the risk of RSA in couples and

fertile controls. The study group comprised of 113 couples with a history of three or more consecutive spontaneous abortions of unexplained etiology before 24th week of gestation. The couples were ascertained through the Institute of Medical Genetics, Department of Obstetrics and Gynaecology. Karyotypes of both men and women with RSA were normal, at a 500 level band of resolution. Women with a history of endocrine or metabolic disorder, autoimmune disease, venous thrombosis or uterine anatomical abnormalities were excluded from the study.

The study sample consisted of two control groups. The first control group consisted of 113 age matched unrelated women, with at least two live births, and no history of pregnancy loss. The second control group consisted of 113 age matched, unrelated, fertile men. Informed consent was obtained from all participants. All patient and control subjects were of Slav origin. The study was approved by the National Ethics' Committee.

Molecular Analysis

Genomic DNA was isolated from peripheral blood leukocytes by a standard procedure using the commercially available kit (Qiagen® FlexiGene kit; QIAGEN GmbH, Hilden Germany). Polymerase chain reaction and RFLP methods were performed to detect the ApaI polymorphism in the 3'UTR of IGF-2 gene and HhaI polymorphism in the sixth CTCF binding site of H19 gene as described previously.[38,39]

Statistical Analysis

Based on the number of cases in the study group (113 couples with RSA) and control subjects (113 healthy women and 113 healthy males), the statistical power was 80% to find a 1.6-fold difference in the frequency of H19 risk genotypes (percentage of carriers in control females and males was set at 31% and 25%, respectively). For IGF-2 risk genotypes (percentage of carriers in control females and males was set at 76% and 44%, respectively), the statistical power to find a 1.3-fold difference in frequency was 80%.

Hardy–Weinberg equilibrium (HWE) was calculated using chi-squared goodness-of-fit test with 10 replicates for simulated *P*-value. Computations were carried out using the VassarStats web site statistical tool (http://faculty.vassar.edu/lowry/VassarStats). A *P*-value 0.05 was considered statistically significant.

Results

No significant deviation from the HWE was observed (for H19 polymorphism $\chi^2 = 29.69$; simulated $P = 0.09$; and for IGF-2 polymorphism $\chi^2 = 29.93$, simulated $P = 0.09$). The genotype and allele frequencies of the IGF-2 ApaI and H19 HhaI polymoprhisms in 113 couples with RSA were compared with those in control groups (113 women and 113 men). There were no differences in the genotype and allele frequencies distribution either for H19 polymorphism between the group of couples with RSA and healthy controls (Table I), or for IGF-2 ApaI polymorphism between female groups (Table II). Statistically significant differences were found in genotype and allele frequencies for IGF-2 ApaI polymorphism in male partners of RSA women and healthy male controls ($\chi^2(2) = 45.12$; $P < 0.0001$) (Table II).

Discussion

Numerous studies on pregnancy mechanisms in the last 50 years have not provided answers, which would improve the understanding and be a potential guide for the therapy of idiopathic RSA. Most studies on the genetic etiology of RSA include only women, but pregnancy, its successful outcome and even

Table I Genotype and Allele Frequencies of H19 HhaI Gene Polymorphism in Recurrent Spontaneous Abortion (RSA) Couples and Controls

	Females				Males			
	RSA (n = 113)		Controls (n = 113)		RSA (n = 113)		Controls (n = 113)	
Genotypes	*n*	%	*n*	%	*n*	%	*n*	%
CC	37	32.8	31	27.4	35	31	31	27.4
CT	44	38.9	47	41.6	51	45.1	53	46.9
TT	32	28.3	35	31	27	23.9	29	25.7
$\chi^2(2)$	0.76				0.35			
P	0.68				0.84			
Alleles	*n*	%	*n*	%	*n*	%	*n*	%
C	118	52.2	109	48.2	121	53.6	115	50.9
T	108	47.8	117	51.8	105	46.4	111	49.1

Table II Genotype and Allele Frequencies of IGF-2 ApaI gene Polymorphism in Recurrent Spontaneous Abortion (RSA) Couples and Controls

	Females				Males			
	RSA (n = 113)		Controls (n = 113)		RSA (n = 113)		Controls (n = 113)	
Genotypes	n	%	n	%	n	%	n	%
AA	16	14.2	27	23.8	15	13.3	63	55.7
AG	49	43.3	43	38.1	54	47.8	28	24.8
GG	48	42.5	43	38.1	44	38.9	22	19.5
$\chi^2(2)$	3.48				45.12			
P	0.17				<0.0001			
Alleles	n	%	n	%	n	%	n	%
A	81	35.8	97	42.9	84	37.2	154	68.1
G	145	64.2	129	57.1	142	62.8	72	31.9

miscarriage are the results of a 'genetically determined conflict' involving three genomes (embryo *versus* mother *versus* father) at the feto-maternal interface.

In this study, we examined the association between IGF-2 ApaI and H19 HhaI polymorphisms in couples with RSA of unknown etiology. While there was no difference between groups for the latter, we found a statistically significant difference in the distribution of IGF-2 ApaI polymorphism frequencies between males from RSA couples and males from healthy couples. Our results indicate that the presence of IGF-2 ApaI G allele may be a risk factor for RSA susceptibility.

During the process of spermatogenesis and oogenesis, germline methylation imprints must be erased so that the germ cell genomes can become maternalized and paternalized depending on germ cell sex.[40,41] In the case of IGF-2 gene, which is expressed only from paternal allele, the active allele in the zygotic genome will be the one derived from the paternal genome. Our results might indicate that paternal transmission and subsequently the expression of the paternal IGF-2 ApaI G allele in zygotic genome contributes to RSA susceptibility, as there were no differences in the distribution of the IGF-2 ApaI polymorphism between both female groups.

The regulation of human IGF-2 gene expression is complex and involves both transcriptional and post-transcriptional control mechanisms. ApaI is a functional polymorphism located in the 3′ non-translated region and as it was shown, it is strongly associated with the transcription of IGF-2 and its level of expression. Various studies have shown associations between the presence of IGF-2 ApaI G allele and IGF-2 mRNA and IGF-2 plasma levels.[42–45] However, to our knowledge, there are no studies on the analysis of placental expression of IGF-2 or other imprinted genes in RSA.

Most of the histopathological studies of conception products from women with RSA have shown that many miscarriages are associated with reduced trophoblast invasion into the decidua and spiral arteries, fragmentation of trophoblast shell, and limited trophoblast proliferation, which led to aberrant angiogenesis and pregnancy arrest.[46,47] As IGF-2 ApaI is a functional polymorphism, it could be speculated that it alters the level of expression that would in turn affect proper functions of IGF-2, mainly those associated with implantation, angiogenesis and placental function. The highest levels of IGF-2 expression are seen in the most proliferative cells of early human placenta and thus, IGF-2 is involved in a variety of functions which regulate placental development, including trophoblast invasion,[48] angiogenesis,[49–51] nutrient transfer,[11] and inhibition of apoptosis.[52]

Another mechanism that could lead to improper gene function and altered gene dosage is disruption of systems involved in the correct identification and maintenance of 'genomic imprinting' that could have deleterious effects on cellular function.[53] The C/T transition detected by H19 HhaI polymorphism suppresses one CpG dinucleotide in the critical DMR region that controls the IGF-2/H19 imprinting status and can lead to loss of imprinting and biallelic expression.[54] We examined the H19 HhaI polymorphism, but no significant difference was found between RSA couples and control groups.

Several limitations of this study should be considered when interpreting and generalizing the results. The relatively small sample size substantially decreased the statistical power, reducing our ability to detect the true effect size. On the other hand, genetic association studies are prone to beta statistical error and populations' specific genotype effects, which make results difficult to reproduce. Further studies are needed to evaluate the implication of IGF-2 gene and its regulation genes, as well as the implication of genomic imprinting and male genome in the etiology of RSA.

In conclusion, the presence of IGF-2 ApaI polymorphism in partners of RSA women could affect

IGF-2 level of expression in placenta and embryo and lead to RSA. Our results indicate that genetic variation linked to epigenetic mechanisms might be associated with a predisposition to RSA.

Acknowledgments

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