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Original Article

Two new GATA-4 mutations were found in chorionic villous samples from recurrent spontaneous abortion patients

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Abstract: The most likely pathogenic mechanism behind recurrent spontaneous abortion is a multifactor mode of inheritance. Since the fetal causes are usually ignored, it has been almost impossible to show that any treatment has an effect. The fetal causes of embryo loss include structural malformations that are incompatible with life and chromosomal aberrations at least. However, the latter is not considered to be the case with recurrent miscarriage. The heart is the first functional organ formed during higher vertebrate development. Cardiac abnormalities may have the most embryonic lethality in all of the developmental defects. GATA-4 is by far the most extensively studied gene controlling embryogenesis, and it is thought to have a dominant role in heart development. Since numerous mutations in GATA-4 have been recognized in a wide range of congenital heart diseases, we hypothesized that some mutations in the GATA-4 gene of the chorionic villous result in lethal cardiac defects and constitute the primary cause of death in the uterus. We screened GATA-4 in 50 chorionic villous samples from recurrent spontaneous abortion patients by direct sequencing. Two novel mutations (c.-156 A>C; c.998-1 G>A) were identified. These two mutations were not found in 60 unrelated chorionic villous samples from induced abortion subjects. As the c.-156 A>C was located in the 5' untranslated region, we speculate that it influences the expression of GATA-4 protein. And the c.998-1 G>A mutation was against to the "GT-AG rule" of splicing. These discoveries may lead to the identification of new genes involved in recurrent spontaneous abortion.

Keywords: GATA-4, chorionic villous samples, recurrent spontaneous abortion, mutation

Introduction

Recurrent spontaneous abortion (RSA) is a disease defined by two or more failed pregnancies. When the cause is unknown, each pregnancy loss merits careful review to determine whether specific evaluation may be appropriate [1] the causative factors can be separated into maternal and fetal aspects. Concerning the maternal factors, established causes of recurrent miscarriage include: uterine anomalies, antiphospholipid syndrome, possible hereditary thrombophilias, alloimmunity factors, infections, endocrine abnormalities and even psychological distress [2]. The fetal causes of embryo loss contain but not confine to structural malformations that are incompatible with life and chromosomal aberrations.

The overall incidence of fetal chromosomal aberrations varies between 25% and 57%, and they are responsible for 70% of aporadic abortions, but the incidence of chromosomal aberrations decreases as the number of miscarriages increases, accounting for considerably smaller fraction of recurrent spontaneous abortion [3]. Most of these chromosome abnormalities represent nonviable defects, such as trisomies 3, 6, 8, 10, 12, 14, 16, and 20, and their presence explains the minimal embryonic development defects observed embryoscopically. Therefore, in a significant proportion of cases in which maldevelopment is similar to that resulting from the trisomies listed above, the underlying cause of this problem is largely ill defined other than for chromosomal abnormalities. Embryonic development is a precisely cho-

reographed event of programmed developmental steps requiring many genes to regulate growth and morphogenesis, and thus the fetal (placental) genome would represent the ideal RSA case for genetic and genomic studies [3]. The heart is the first functional organ formed during higher vertebrate development. A beating heart is required to facilitate the exchange of nutrients between mother and fetus. As the most prevalent defects reported at birth, cardiac abnormalities may cause the most embryonic lethality in all of the developmental defects.

The zinc finger transcription factor GATA-4 is essential for the beating of cardiomyocytes and even ectopic GATA-4 can induce stem cell to form beating cardiomyocytes [4]. Small changes in the level of GATA-4 protein expression can dramatically influence cardiac morphogenesis and embryonic survive [5]. In humans, heterozygote mutations in GATA-4 are associated with an isolated congenital heart defects (CHDs), of which mendelian and chromosomal syndromes account for less than 20%. Homozygous GATA-4 null mice die *in utero between embryonic days 7.0 and 9.5* due to severe developmental abnormalities in the off spring's heart, lacking a centralized heart tube and foregut, and developing partially outside the yolk sac, which results from a fundamental failure in lateral to ventral folding early in embryogenesis [6]. Moreover, stem cell studies not only prove inhibition of GATA-4 expression in early stage restrains the development of heart precursor cells and end-stage myocardial cells, blocking the formation of original myotube but also to show GATA-4 increases cardiomyocytes survival by enhancing release of VEGF and IGF-1 and preserves mitochondria under hypoxia conditions [7, 8]. The fetus develops within a low-oxygen environment for much of the first trimester, especially before the 8th gestation age, while in clinical cases, 89% of human recurrent miscarriages occur in the first trimester and miscarriages are often diagnosed by ultrasound between 6 and 10 weeks based on the inability to see an embryonic pole and cardiac activity. Based on these observations, we hypothesized that human spontaneous abortion may be related to mutations in the GATA-4 gene of chorionic villous (fetal) during embryogenesis and that these mutations cause embryonic lethality. Thus, the purpose of this study was to explore the association between GATA-4 and RSA by determining mutations of the GATA-4 gene in chorionic villous from RSA patients.

Materials and methods

Subjects

The study involved 50 unrelated RSA patients (age: 22-40 years with a median of 29.1 years) who underwent dilation and curettage for missed abortion in the First Affiliated Hospital of Medical College, Sun Yat-sen University (Guangzhou, China) during the period of September 2009 to October 2015. All patients had histories of at least two successive missed abortions between the 6th and 12th weeks of gestation, and there was no successful pregnancy record (with the same partner). The median number of miscarriages was 3.1. Missed abortions were diagnosed by transvaginal ultrasound and included only embryonic demises, where an embryonic pole was identified without cardiac activity. The gestational age was determined from the last menstrual period (LMP) to the date of curettage. The actual developmental age (DA) of the embryos was derived from their crown-rump length (CRL), measured by ultrasonography. The RSA patients included should meet all the below criteria at the same time [9]: 1) uterus and cervical abnormalities were excluded by pelvic examination, ultrasound and a diagnostic hysteroscopy; 2) Chlamydia and ureaplasma were excluded by Cervical mucus culturing; 3) Chromosome problems were excluded by Karyotypes of abortion couples and abortuses; 4) Luteal function defect, hyperprolactinemia and hyperandrogenemia were excluded by comprehensive hormonal examinations; 5) Endocrine diseases, e.g., diabetes, hyperthyroidism and hypothyroidism were excluded; 6) Autoimmune factors associated with systemic lupus erythematosus (SLE) and the antiphospholipid syndrome (APS) such as antinuclear antibodies (ANA), lupus anticoagulant (LA), and anticardiolipin antibodies (ACL) were tested in three consecutive visits every other month; 7) All male partners had normal semen status. The control population consisted of 60 healthy women without prior miscarriage and stillbirth for induced abortion. The populations were matched for gestational and maternal age. Written consent for the study was obtained from all patients and the control population

Table 1. Primers used for PCR-direct sequencing

Exon	Forward primer	Reverse primer	PCR product	Tm (°C)
1-1	TCATTTGAAGCGTGGAAGAAG	TGGCCAAGCTCTGATACATG	574	56
1-2	TCCCACGCATATTATCGTTGTTGC	ACTCGAGGTAGTGAAGCACCATC	847	54
2	CCCGAGGTGGTCTTCTCTTTC	CTGGATCATTCTGGTGGCTC	478	58
3	CTGATTTATTCCTCGCAGTGG	TGTAAAGGACGGAAGAGGCC	484	58
4	CTTTCTCGCTGAGTTCCAGG	GTCTTTGCAGTCGGCAATG	499	56
5	ACTGTAGCCCTCCGCAGATA	TGAGGCCTGGCTGCAAGTC	404	60
6	ACATCTGCATAGCAGGGCAC	ATGCAGTGTGCTCGTGCTGAAG	516	58

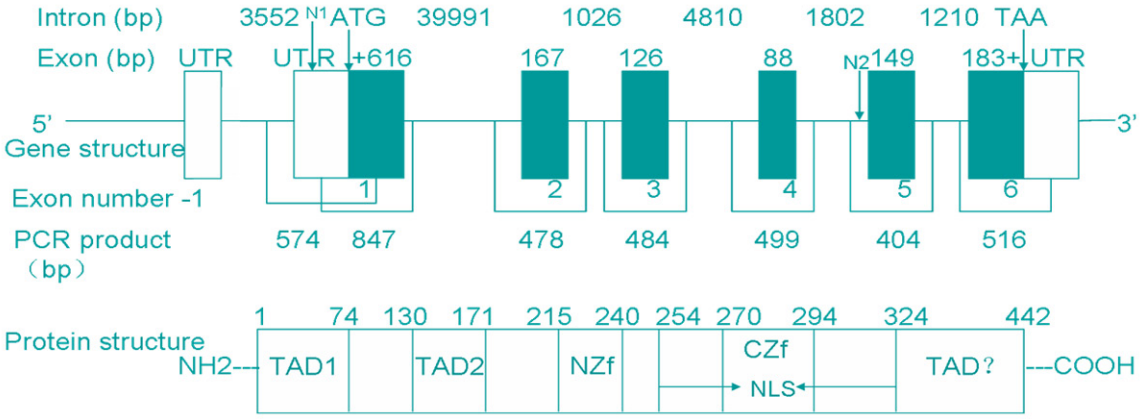

Figure 1. Schematic structure of the human GATA-4 gene and functional structure of GATA-4 protein. The coding sequences, non-coding sequences and the introns were depicted as black boxes, white boxes, and horizontal lines, respectively. Exon numbers were shown below the exon boxes. The sizes of the exons and introns were labeled above them. The new mutation was located in the 5'UTR of GATA-4 gene, N1, N2 represent the location of the mutation. The PCR product and primer design pattern were shown below the exon numbers. Sequence functional chart was attached the last. TAD, NZf, CZf, NLS (254-354) strands for transcriptional activation domain, N-terminal zinc finger, C-terminal zinc finger, nuclear localization signal, respectively.

Table 2. Clinical manifestations between RSA group and control group

Clinical manifestations	RSA group	Control group	*P* value
Maternal age	29.1	28.5	>0.05
Gestational age	8.2	8	>0.05
Number of RSAs	3.1	0	<0.05
Number of induced abortions	0	1.2	>0.05
Number of live children	0	2	<0.05
Number of stillbirth	0	0	
Maternal BMI	20.1	21.3	>0.05

after detailed information sessions with each individual. The study protocol was approved by the Ethics Committee of Sun Yat-sen University.

Tissue samples and DNA extraction

Chorionic villous samples (CVS) were obtained by curettage. Placental villi were separated from the curettages of the abortus using a standardized technique, where the physician carefully separating out the villous tissue and thoroughly washing it with saline without no visible blood and maternal deciduas, as previously described [10], to prevent the maternal deciduas from contaminating the CVS and skewing the results. DNA was extracted from CVS using the AxyPrep TM multisource DNA extraction Mini kit (AXYGEN, Pittsburgh, PA) according to the manufacturer's instructions.

Searching for mutations in GATA-4 gene of chorionic villous samples

Direct-sequencing analysis was carried out for the detection of mutations. To sequence all of the 6 coding exons and flanking intron-exon boundaries as well as the fragments upstream of the first coding exon and downstream of exon 6, respectively, we constructed 7 sets of PCR primers according to human cDNA infor-

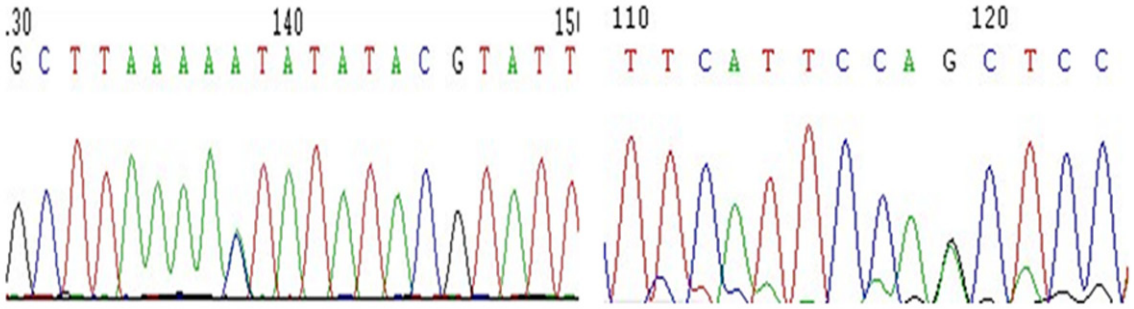

Figure 2. Two mutations identified in GATA-4 in the villous tissues.

mation of the GATA-4 gene (GenBank accession number: NM- 002052) (**Table 1** and **Figure 1**). PCR was performed in a volume of 50 μL, containing 2 μL (20 ng) genomic DNA, 4 μL (20 pmol) each primer, 25 μL Takara Ex Tap Mix and 15 μL ddH_2O. The PCR profile was as follows: initial denaturation at 98°C for 1 min, followed by 35 cycles of 98°C for 30 s, annealing for 30 s, and extension at 72°C for 1 min. PCR products were individually pretreated with a mixture of 1 unit ExoI and 1 unit SAP (TAKARA). Direct dye terminator sequencing of the purified PCR products was conducted using the ABI Prism BigDye system following the manufacturer's (ABI, Foster City, CA). Precipitation was done by 75% alcohol after second purification by 1 unite SAP. HiDi formamide was used in the subsequent denaturation, and finally the samples were subjected to sequencing using an ABI PRIMA 3100 Genetic Analyzer. The results were analyzed by the Sequence Scanner and DNASTAR software. The GATA4 mutations identified were confirmed by reverse sequencing of the independent PCR amplifications using the mutation carrier case DNA samples.

Statistical analysis

The data in the **Table 2** are shown by Mean values; all data were analyzed with T test by the SPSS 13.0 software package (SPSS, Chicago, IL, USA). Difference was considered significant when $P<0.05$ while difference was not statistically significant when $P>0.05$.

Result

Clinical characteristics between RSA group and control group

The clinical characteristics of the women included in the study both cases and controls had been sorted in **Table 2**. There was no difference found in the maternal age, gestational age, number of induced abortions and maternal BMI between RSA group and control group. In RSA group, all women included had no induced abortion, live children while women having induced abortions had no history of spontaneous abortion. All women of the two groups had no stillbirth.

Note: The data in the **Table 2** are shown by Mean values; difference was considered significant when $P<0.05$ while difference was not statistic significant when $P>0.05$.

Sequence results

No mutation in the coding region of GATA-4 was found. However, the sequence variation, a A to C substation at position -156 (156 bp 5' from the initiation codon, c.-156 A>C) was identified within the GATA-4 5'UTR of exon 1 in two chorionic villous samples from RSA patients. Another variant was located in the 4^{th} intron found in one chorionic villous sample from RSA patients, referred to as c.998-1 G>A, 1 bp 5' from exon 5. The two mutations were never observed in 60 control GATA-4 gene (**Figure 2**).

Bioinformatic analyses of the two mutations

Analysis of RNA or DNA second structure: We have used the RNA-fold web severe to predict the possible change of the second structure before and after mutation for the two mutations. As we expected, the second structure of 5' untranslated region (5'UTR) changed significantly when mutation N1 c.-156 A>C happened both by MFE and centroid structure (**Figures 3**, **4**). We also used the RNA-fold web server to predict the second structure of DNA for the whole forth intron and the fifth exon to evaluate the possible effect of N2 mutation. Similarly, the second structure was slightly different before and after the mutation.

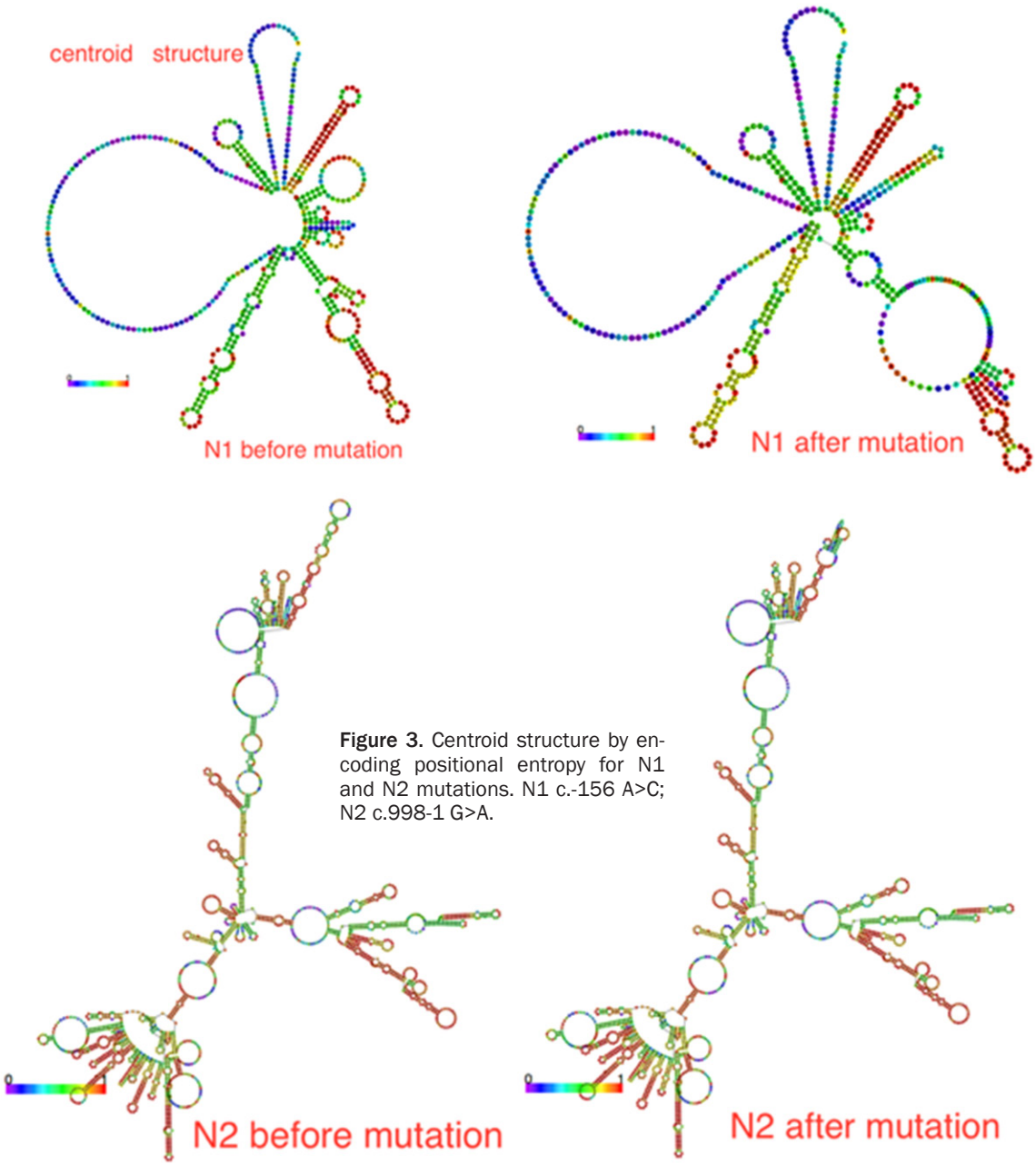

Figure 3. Centroid structure by encoding positional entropy for N1 and N2 mutations. N1 c.-156 A>C; N2 c.998-1 G>A.

Analysis of splice site: The N2 mutation was located at the splice acceptor site in the forth intron and was against to the "GT-AG" splice rule, we had used the NetGene2 software to predict the mutation of N2 on splice site. As we speculated, when the base changed from G to A, there would be lack of a possible acceptor splice site (**Figure 5**).

Discussion

Recurrent spontaneous abortion (RSA) continues to be a challenging reproductive problem for the patient and clinician. The most likely pathogenic mechanism behind RSA is a multi factorial mode of inheritance. The maternal causes are well known while the fetal causes are usually ignored. Hence, it has been almost impossible to show if any treatment has an effect [11].

Approximately 70% of aborted conceptions with sporadic spontaneous abortion reveal some chromosome abnormality. However, the majority of spontaneous abortions with embry-

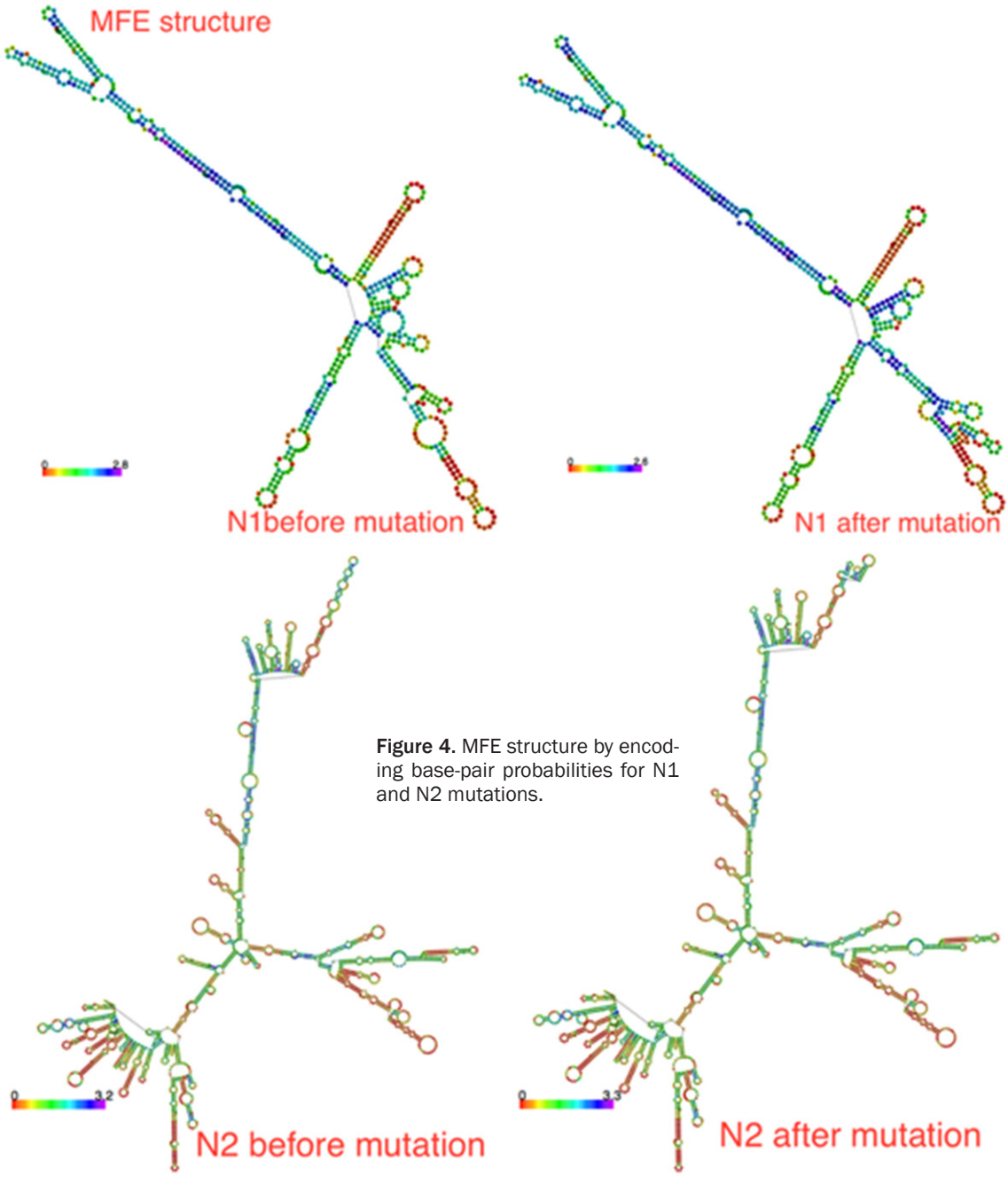

Figure 4. MFE structure by encoding base-pair probabilities for N1 and N2 mutations.

onic abnormalities occur by chance and this is not considered to be the case with recurrent miscarriage. In the present study, we excluded anembryonic gestations, defined by no embryonic pole seen on ultrasound at 7 weeks gestation, as anembryonic gestations usually result from rare trisomies such as trisomies 3, 6 and 12. Moreover, all the CVS were karyotyped to exclude chromosomal anomalies.

Morphological examination of the conceptus consists of gross and microscopic examination of the gestational sac and the embryo and sampling for cytogenetic studies. Currently, the only method that allows direct visualization of the embryo *in utero* is embryoscopy. Indeed, Philipp et al performed embryoscopy on missed abortions just prior to curettage and found developmental defects in 200/233 missed abortions (85%) [12]. Meanwhile, they karytyped 221 embryos, of which only 56 (25%) were eukaryotypic. The factors responsible for embryonic maldevelopment with a normal karyotype are presently unknown. Monozygotic twins concor-

Acceptor splice sites, direct strand

before mutation

pos 5'->3'	phase	strand	confidence	5' intron exon 3'
115	1	+	0.07	GTGGTGACAG^CATCGGACAT
139	1	+	0.30	GGCCTTTCAG^GACAGGATGA
144	0	+	0.41	TTCAGGACAG^GATGAAGAGC
151	1	+	0.07	CAGGATGAAG^AGCCCAGCAA
153	0	+	0.07	GGATGAAGAG^CCCAGCAAAA
680	2	+	0.56	TAATTTCTAG^ATTAAGAATA
910	1	+	0.17	TTTCTGACAG^AACAACGGCT
1158	2	+	0.33	CTTCTGGCAG^GCATATTTCC
1278	1	+	0.29	TCTCACGTAG^CAATCACAGA
1467	2	+	0.27	TTCATTATAG^AGTATTATCT
1739	0	+	0.30	TGTCTCGCAG^GCTGCCGGCT
1801	1	+	0.23	TTCATTCCAG^CTCCTTCAGG
1810	1	+	0.33	GCTCCTTCAG^GCAGTGAGAG
1814	2	+	0.07	CTTCAGGCAG^TGAGAGCCTT
1818	0	+	0.17	AGGCAGTGAG^AGCCTTCCTC
1820	2	+	0.18	GCAGTGAGAG^CCTTCCTCCC
1835	2	+	0.34	CTCCCGCCAG^CGGTGCTTCC
1847	2	+	0.44	GTGCTTCCAG^CAACTCCAGC

Acceptor splice sites, direct strand

after mutation

pos 5'->3'	phase	strand	confidence	5' intron exon 3'
115	1	+	0.07	GTGGTGACAG^CATCGGACAT
139	1	+	0.30	GGCCTTTCAG^GACAGGATGA
144	0	+	0.41	TTCAGGACAG^GATGAAGAGC
151	1	+	0.07	CAGGATGAAG^AGCCCAGCAA
153	0	+	0.07	GGATGAAGAG^CCCAGCAAAA
680	2	+	0.56	TAATTTCTAG^ATTAAGAATA
910	1	+	0.17	TTTCTGACAG^AACAACGGCT
1158	2	+	0.33	CTTCTGGCAG^GCATATTTCC
1278	1	+	0.29	TCTCACGTAG^CAATCACAGA
1467	2	+	0.27	TTCATTATAG^AGTATTATCT
1739	0	+	0.31	TGTCTCGCAG^GCTGCCGGCT
1810	1	+	0.31	ACTCCTTCAG^GCAGTGAGAG
1814	2	+	0.07	CTTCAGGCAG^TGAGAGCCTT
1818	0	+	0.17	AGGCAGTGAG^AGCCTTCCTC
1820	2	+	0.18	GCAGTGAGAG^CCTTCCTCCC
1835	2	+	0.34	CTCCCGCCAG^CGGTGCTTCC
1847	2	+	0.44	GTGCTTCCAG^CAACTCCAGC

Figure 5. Possible splice sites of the whole intron 4 and exon 5 before and after mutation.

dant for major developmental defects are a strong indicator of a single gene defect with a high risk of recurrence in future pregnancies [13].

The GATA family of transcription factors comprises six members, which can be subdivided in to two subfamilies based on sequence similarity and expression profiles. GATA-1, -2 and -3 are involved in hematopoeisis and ectodermal patterning, while GATA-4, -5 and -6 are expressed in cardiac tissue and endodermal derivatives [14]. Of the three cardiac GATAs, GATA-4 is by far the most extensively studied and is thought to have a dominant role in heart development. Numerous mutations in GATA-4 have been recognized in a wide range of cases, including tetralogy of Fallot (TOF), pulmonary stenosis, atrial septal defects (ASDs), ventricular septal defects, atrioventricular defects and patent ductus arteriosus [15, 16]. Recently clinical study has showed genetic variations (SNPs) in GATA-4 can influence on the coumarin maintenance [17]. These findings prompted us to explore the significance of the GATA-4 gene in the embryo in the predisposition to RSA. In the present study, no mutation was found in the six exon segments in GATA-4 from the villous tissue of embryos. However, one variant at location c.-156 A>C (NG-008177:g 3950 A>C) was found in the 5'-UTR of GATA-4 in two RSA villous tissue samples. As the variant was located in the 5' untranslated region (5'UTR), we speculate that this variant may modulate the expression of GATA-4 gene at the transcriptional and translational stages. Individuals with A/G genotype may ultimately have had a decreased expression of GATA-4 protein. Direct in vitro experiments have not been performed to test the effect of the variants on level of mRNA or protein expression, we had used the RNA-fold web severe (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) to predict the effect of mutation N1 on the second structure of 5'UTR. To our surprise, the second structure indeed had significantly changed when mutation N1 happened both in centroid (**Figure 3**) and MFE structure (**Figure 4**). It had been proved that mRNA second structure region effect the efficiency of translation initiation in the late twenties century, so we speculated that the N1 mutation may change the efficiency of translation initiation and thus play an important role in the occurrence of RSA.

Analysis of genomic DNA from another patient revealed a G to A base substitution in the intron 4 at location c.998-1 G>A (NG_008177:g.52727 G>A). As the mutation is against to the "GT-AG rule" of splicing, it may give rise to a new GATA-4 transcript or an unstable transcript quickly destroyed after transcription. To prove this, we had both used the RNA-fold web-server and NetGene2 software (http://www.cbs.dtu.dk/services/NetGene2/) to predict its effect on pre-mRNA or DNA second structure and splice site. Mild change of second structure was observed, and the real splice site "TTCATTCCAG^CTCCTTCAGG" was missed when N2 mutation happened. This can be further proved by gene-specific mRNA analysis and directly sequencing the cDNA in the future. At

the same time, there were literature reporting that mutations at the splice donor site can influence the translational efficiency of mRNA transcripts [18], whether the GATA-4 protein was less efficiently produced from the A type transcript than the G type transcript when the N2 mutation (G⟶A) locating in splice acceptor site happened still deserves to be further researched.

It was very important to test the association at the mRNA or protein level between the above mutations and RSA. Unfortunately, we cannot do this as we just sort the genomic DNA sample. Future research can be performed by the luciferase reporter gene assay in a relevant cell linein vitro to test its real relation. Another weakness of the findings we must acknowledge is that 2 of 50 RSA vs 0 of 60 control samples do not have sufficient power to show a difference. So a larger number of control subjects are very necessary in future research in order to show an association.

In conclusion, we identified two new GATA-4 mutations in the villous samples, suggesting that it likely has a role in the pathogenesis of RSA. Studies on the impact of these GATA-4 mutations with RSA and the five other GATA transcription factors will be important to define in the future.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

GATA-4, GATA binding protein 4; RAS, recurrent spontaneous abortion; CHDs, congenital heart defects; CVS, chorionic villous samples; TAD, transcription activation domain; NZF, N-terminal zinc finger; CZF, C-terminal zinc finger; NLS, Nuclear localization signal.

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