Chromosomal karyotype in chorionic villi of recurrent spontaneous abortion patients

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Summary   **Recurrent spontaneous abortion (RSA) is a multifactorial disease of which the exact causes are still unknown. In the current study, we aimed to analyze the distribution of abnormal embryonic karyotypes in RSA. 781 RSA patients of 17 hospitals in Shanghai from January 2014 to September 2016 were enrolled. Fetal villus tissues were collected during uterine curettage and then cultured in situ for karyotyping. All of the 781 cases were successfully cultured. There were 393 cases of abnormal karyotypes, accounting for 50.3% of the total cases. Women with abnormal embryonic karyotype were significantly older compared to those with normal karyotype ($P < 0.001$). The majority of patients with abnormal karyotype fell among age groups of 25-29 and 30-34. There were 247 cases of aneuploidy, accounting for 62.8% of the total abnormal karyotype cases. Autosomal trisomy was the primary form of aneuploidy (189/247, 76.5%), and the most common types were trisomy-16 ($n = 69$), trisomy-22 ($n = 28$), trisomy-21 ($n = 21$), trisomy-15 ($n = 15$), and trisomy-13 ($n = 10$). Abnormal karyotype is a major factor related to RSA. Further studies are needed to elucidate the etiology of RSA in order to achieve more effective prevention and treatment.**

Keywords: Recurrent spontaneous abortion (RSA), fetal chorionic villi, karyotype, chromosomal abnormality

1. Introduction

Recurrent spontaneous abortion (RSA), also known as recurrent miscarriage, habitual abortion or recurrent pregnancy loss (RPL), is usually defined as at least two consecutive pregnancy losses prior to the 20th gestational week of pregnancy (*1*). The probability of couples of childbearing age affected by RSA has been estimated to approach 2-5%, and it varies according to different definitions and criteria (*2*). Most of these abortions occur prior to the 10th gestational week of pregnancy (*3*). The etiology of RSA has not been well elucidated

and may be multifactorial, as previous research has demonstrated that several factors may play roles in RSA, including uterine anomalies, chromosomal abnormalities in either partner, antiphospholipid syndrome (APS), thrombophilic disorders, endocrine factors, microbial infections, maternal diseases, and male factors such as sperm deformation and DNA fragmentation (*1,2,4*).

The majority (50-60%) of early pregnancy losses are caused by chromosomal abnormalities, which are either of parental origins or *de novo* abnormalities from parents with normal karyotypes (*5,6*). Embryonic aneuploidy, which increases significantly with advanced maternal age, accounts for a large portion of spontaneously abortus (*5*).

Previous studies have suggested that abnormal embryonic karyotype may contribute to RSA (*7-11*). *De novo* numerical abnormalities, particularly autosomal trisomies, may explain a proportion of RSA (*10*). There

is also a notion that most chromosomal abnormalities occur *de novo*, probably led by random errors during gametogenesis (*12*). The current study aimed to analyze the distribution of abnormal embryonic karyotypes in RSA using the next generation sequencing techniques (NGS). The method of NGS enables detailed analysis of the entire genomic makeup of the fetus based on either a biopsy sample from the fetus directly or a blood sample from the pregnant women. Due to its high resolution, this method can provide information down to the smallest detail. Also, the broad coverage of this method enables obstetricians and gynecologists to identify even the very rare diseases associated with certain karyotypes. With the help of different databases (such as DECIPHER and ISCA), it is now possible to make more accurate diagnoses as well as predict prognoses for patients. Compared with the traditional chromosomal karyotyping method, NGS has obvious advantages in accuracy and coverage, therefore providing more detailed information about the sample, including etiology, risk stratification, molecular diagnosis and prognosis.

2. Materials and Methods

2.1. *Study population*

Patients who sought treatment for RSA between January 2014 and September 2016 at the following 17 hospitals in Shanghai were enrolled: Obstetrics and Gynecology Hospital of Fudan University, Shanghai First Maternity and Infant Hospital, Zhongshan Hospital, Renji Hospital of Shanghai Jiaotong University School of Medicine, Shuguang Hospital, Yueyang Hospital, Shanghai General Hospital, Shanghai Sixth People's Hospital, Shanghai Eighth People's Hospital, Shanghai Ninth People's Hospital, Shanghai Pudong Hospital, Central Hospital of Minhang District, Changning Maternity and Infant Health Hospital, Putuo Maternity and Infant Health Hospital, Jiading Maternity and Infant Health Hospital, Maternity and Infant Health Hospital of Pudong New District, Shanghai Institute of Planned Parenthood Research Hospital. A total of 781 patients were recruited for the current study.

The study protocol conformed to the ethical guidelines of the 2000 Declaration of Helsinki and was approved by the institutional review board at Obstetrics and Gynecology Hospital of Fudan University. All participants provided written informed consent.

2.2. *DNA preparation and sequencing*

Villus samples were collected from the aborted tissue. DNA from these samples was extracted using the QIAamp DSP DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The extracted DNA was then digested using NEBNext dsDNA Fragments (NEB).

Library construction, quality control, and pooling were performed according to instructions of JingXin Fetal Chromosome Aneuploidy (T21, T18, T13) Testing Kits (CapitalBio Corporation, China). For DNA sequencing, 15~20 libraries were pooled and sequenced with ~200 bp reads using JingXin BioelectronSeq 4000 System semiconductor sequencer (CapitalBio Corporation).

2.3. *Data extraction*

Reads were aligned to the human genomic reference sequences (hg19) using the BWA (*30*). Reads, which were unmapped or had multiple primary alignment records were filtered by FLAG field in the alignment file, using an in-house Perl script. Duplicate reads were identified by Picard (*http://picard.sourceforge.net/*) and removed by an in-house Perl script. The remaining reads were considered unique reads for further analysis. To eliminate the effect of GC bias, we applied an integrated method for GC correction using a three-step process (LOESS regression, intrarun normalization, and linear model regression) according to Liao's paper (*13*). Combining the Z scores of adjacent 1Mb blocks would increase the precision to detect subchromosome aberrations using Stouffer's Z-score method (*14*). When Stouffer's Z score is larger than 5, we classify it as microduplication, whereas when it is less than -5, we classify it as microdeletion.

2.4. *Statistical analysis*

Age was compared using Student's *t* test as a continuous variable and Chi-square test as a categorical variable. All above analyses were two-sided and performed using EXCEL2007 and SPSS 16.0 (SPSS, Inc., Chicago, IL). A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. *The demographics of patients*

Of the 781 enrolled subjects, 388 (49.7%) abortuses had normal karyotype and 393 (50.3%) had abnormal karyotypes. Patients were from almost all parts of China, while most of them resided in metropolitan Shanghai, Zhejiang province, and Jiangsu province (Supplementary Table 1).

The age distribution of the subjects is shown in Table 1, and women with abnormal karyotypes were significantly older compared to women with normal karyotype (*P* < 0.001). Figure 1 shows the distribution of abnormal karyotypes among different age categories. The prevalence of abnormal embryonic karyotypes increased with age after age 25, although there was no significant difference detected across all age categories (*P* = 0.136).

Table 1. Age distribution of two groups

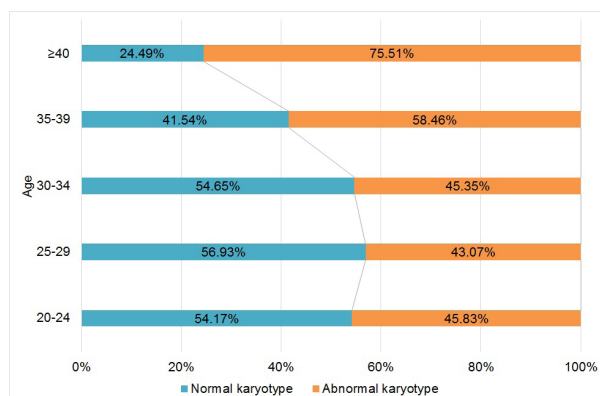| Age	Normal karyotype (n = 382)	Abnormal karyotype (n = 364)	P
Age (year), mean ± SD	30.67 ± 4.03	32.23 ± 5.02	< 0.001
Age (year), median (range)	30 (21, 45)	32 (20, 45)	
Age category, n (%)			< 0.001
20-24	13 (3.4%)	11 (3.0%)	
25-29	156 (40.8%)	118 (32.4%)	
30-34	147 (38.5%)	122 (33.5%)	
35-39	54 (14.1%)	76 (20.9%)	
≥ 40	12 (3.1%)	37 (10.2%)	

Figure 1. Distribution of embryonic karyotypes among different age categories. Among 24 women aged 20-24, the proportion of abnormal embryonic karyotype was 45.83%, and the normal embryonic karyotype proportion was 54.17%. Among 274 women aged 25-29, the percentages of abnormal karyotypes and normal karyotypes were 43.07% and 56.93%, respectively. Among 269 women aged 30-34, the abnormal karyotype proportion was 45.35%, and the normal karyotype was 54.65%. Among 130 women aged 35-39, the abnormal karyotype versus normal karyotype proportion was 58.46% versus 41.54%. Among 49 women aged over 40, the proportion of abnormal karyotype was 75.51%, and the normal karyotype proportion was 24.49%.

3.2. *The details of embryonic abnormalities*

Details of embryonic abnormalities are presented in Table 2. The most frequent type of chromosomal abnormalities was aneuploidy (247/393, 62.8%), followed by structural abnormalities (83/393, 21.1%), unbalanced structural abnormalities (45/393, 11.5%), mosaicism not involving sex chromosomes (12/393, 3.1%), and sex chromosome mosaicism (6/393, 1.5%).

Most patients with abnormal karyotypes fell among age groups 25-29 and 30-34 (Table 3). The average age for women with aneuploidy, structural abnormalities, and mosaicism (involving both sex chromosomes and autosomes) was 32.59 ± 4.86, 30.68 ± 4.53, and 32.11 ± 6.37, respectively. The distribution profile of abnormal karyotypes varied slightly among different age categories (Figure 2). Aneuploidy was still the most common form of abnormal karyotype among all age categories. The highest aneuploidy rate was among women between 20 and 24 years of age (72.7%), and the lowest rate occurred in those aged 25-29 (55.1%). Structural abnormalities were the second most common form of abnormal

karyotype among age groups 25-29, 30-34, and 35-39, while other karyotype abnormalities sharply increased in those aged over 40. The frequency of mosaicism stayed stable across different age groups (Figure 2).

Most of the fetuses with aneuploidy presented autosomal trisomy (189/247, 76.5%). Other types of aneuploidy included autosomal monosomy (6/247, 2.4%), autosomal double trisomy (13/247, 5.3%), Turner syndrome (45, XO) (30/247, 12.1%), Triple X syndrome (2/247, 0.8%), Jacob's syndrome (2/247, 0.8%), and Triploidy 69XXY (5/247, 2.0%). The most common types of autosomal trisomies in our group of fetuses were trisomy-16 (69/189, 36.5%), trisomy-22 (28/189, 14.8%), trisomy-21 (21/189, 11.1%), trisomy-15 (15/189, 7.9%), and trisomy-13 (10/189, 5.3%) (Table 2). Trisomy-16 was the most common type of autosomal trisomy among women between 25 and 39 years of age, and trisomy-22 was commonly found in age group 30-34 (Figure 3).

We also presented selected karyotypes (Table 4) with known clinical indications such as microduplication syndromes and microdeletions.

4. Discussion

Miscarriage is clinically recognized in 10-20% of pregnancies. Recurrent spontaneous abortion (RSA) is defined as two or more pregnancy losses, which is diagnosed clinically by ultrasonography or histopathologic examination (*1*). There is about 5% of women going through two consecutive spontaneous abortions, and the proportion is even higher in those over 35 years old. Less than 1% of women are affected by three consecutive spontaneous abortions (*3*). Despite the long debate about the exact definition among different international societies, RSA is an important health issue (*15-17*). With the implementation of second child policy in China, many couples are now having difficulty getting pregnant or facing the problem of pregnancy loss when expecting a second baby, thus the issue of RSA will continuously affect an increasing number of couples. Therefore, it is essential to investigate the etiology of RSA for complete evaluation and targeted treatment provided to couples with a history of RSA during a subsequent pregnancy.

The causes of RSA are complicated, involving genetics, uterine anomalies, hormonal or metabolic

Table 2. Karyotypes of 781 RSA patients

Karyotype	*n*	% of total patients	% of patients with abnormal karyotype
Normal karyotype	388	49.7%	
Abnormal karyotype	393	50.3%	
Aneuploidy			
Autosomal monosomy	6	0.8%	1.5%
Autosomal trisomy			
47, XN,+2	6	0.8%	1.5%
47, XN,+3	3	0.4%	0.8%
47, XN,+4	6	0.8%	1.5%
47, XN,+5	2	0.3%	0.5%
47, XN,+6	4	0.5%	1.0%
47, XN,+7	6	0.8%	1.5%
47, XN,+8	3	0.4%	0.8%
47, XN,+9	2	0.3%	0.5%
47, XN,+10	1	0.1%	0.3%
47, XN,+11	1	0.1%	0.3%
47, XN,+12	2	0.3%	0.5%
47, XN,+13	10	1.3%	2.5%
47, XN,+14	3	0.4%	0.8%
47, XN,+15	15	1.9%	3.8%
47, XN,+16	69	8.8%	17.6%
47, XN,+17	1	0.1%	0.3%
47, XN,+18	5	0.6%	1.3%
47, XN,+20	1	0.1%	0.3%
47, XN,+21	21	2.7%	5.3%
47, XN,+22	28	3.6%	7.1%
Autosomal double trisomy	13	1.7%	3.3%
Turner syndrome (45,XO)	30	3.8%	7.6%
Triple X syndrome (XXX)	2	0.3%	0.5%
Jacob's syndrome (47,XYY)	2	0.3%	0.5%
Triploidy 69XXY	5	0.6%	1.3%
Structural abnormalities			
Deletion	16	2.0%	4.1%
Duplication	54	6.9%	13.7%
Unbalanced translocation	13	1.7%	3.3%
Mosaicism not involving sex chromosomes	12	1.5%	3.1%
Sex chromosome mosaicism	6	0.8%	1.5%
Unbalanced structural abnormalities	45	5.8%	11.5%

Table 3. Major karyotype abnormalities by age categories

Age category (year)	Aneuploidy (*n*, %)	Structural abnormalities (*n*, %)	Mosaicism* (*n*, %)	Other (*n*, %)	Total (*n*)
20-24	8 (72.7%)	1 (9.1%)	1 (9.1%)	1 (9.1%)	11
25-29	65 (55.1%)	35 (29.7%)	5 (4.2%)	13 (11.0%)	118
30-34	82 (67.2%)	23 (18.9%)	7 (5.7%)	10 (8.2%)	122
35-39	53 (69.7%)	13 (17.1%)	3 (4.0%)	7 (9.2%)	76
40 +	24 (64.9%)	2 (5.4%)	2 (5.4%)	9 (24.3%)	37
Missing	15 (51.7%)	9 (31.0%)	0 (0%)	5 (17.2%)	29

*Mosaicism: involving both sex chromosomes and autosomes.

disorders, infection, reproductive immunity, and thrombophilias. Genetic factors include abnormal karyotypes, mutations, genetic polymorphisms, and so on. Previous studies have suggested that abnormal embryonic karyotype is one of the most common causes of RSA. Abnormal karyotypes contribute to the majority of miscarriages, accounting for about 51% in patients with RSA and up to 76.3% in women with sporadic abortion (*18*). Consistent with other studies (*10,19*), we observed that about half of our RSA abortus had abnormal karyotypes (393/781, 50.3%). In addition,

previously diagnosed abnormal embryonic karyotypes can be a predictor of subsequent miscarriages. It was reported that for patients who had been diagnosed with abnormal embryonic karyotype in the first determination, 76.2% of them had a subsequent abnormal embryonic karyotype (*5*).

The common types of chromosomal abnormalities include aneuploidy, structural abnormalities, unbalanced structural abnormalities, and mosaicism. It is reported that in older women (over 35 years old) with RSA, the majority of miscarriages are caused by fetal
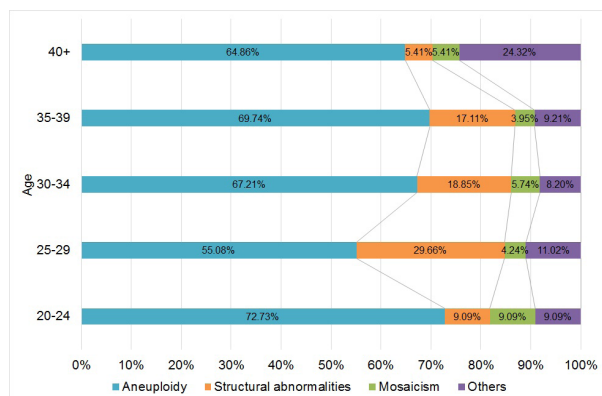
Figure 2. Distribution profile of abnormal karyotypes among different age categories. Comparison of the distribution profile of abnormal karyotypes among different age categories. *Mosaicism: involving both sex chromosomes and autosomes.
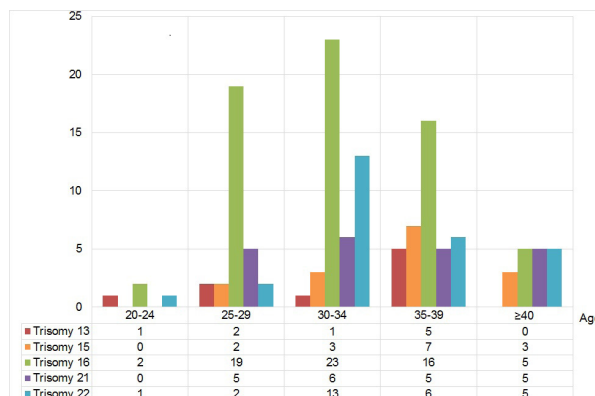

Figure 3. Distribution profile of autosomal trisomies among different age categories. Comparison of the distribution profile of autosomal trisomies among different age categories.

Table 4. Selected karyotypes with known clinical indications

No.	Karyotype	Indications
1	46,XY,del(7)(q11.23)	Chromosome 7q11.23 deletion syndrome
2	46,XY,del(8)(p23.3p21.2)	Chromosome 8p23.1 deletion syndrome. Deleted fragments contain many genes such as *ZNF596* and *FBXO25*.
3	46,XY,del(8)(p23.3p11.23)	Chromosome 8p deletion syndrome
4	46,XY,del(5)(p15.33p14.3)	Cri du Chat Syndrome -5p deletion. Deleted fragments contain many genes such as *PLEKHG4B* and *LRRC14B*.
5	46,XX,del(22)(q11.23q12.1)	Chromosome 22q11.2 distal deletion syndrome
6	46,XX,del(7)(q36.3)	Acheiropody and preaxial polydactyly, Currarino syndrome, and Holoprosencephaly 3
7	46,XY,del(7)(q11.23)	Chromosome 7q11.23 deletion syndrome
8	46,XN,del(11)(q24.1-q25)	Jacobsen syndrome
9	46,XN,del(Xp22.31).[GRCH37/hg19](6.48Mb-8.14Mb)×1	Steroid sulphatase deficiency (STS)
10	46,XN,dup(5)(q31.1q35.3)	The duplicated fragment includes the *NKX2-5* gene, which is associated with Tetralogy of Fallot and autism.
11	46,XN,dup(X)(q28)	The duplicated fragment includes genes such as *IDS* and *MAGEA8*. Reported cases with this duplication show retarded language development, mental retardation, head and facial deformity, and toe deformity.
12	46,XN,dup(1)(p32.3)	The duplicated fragment includes genes such as *DHCR24*, which is associated with physical and mental retardation.
13	46,XN,dup(14)(q11.2)	The duplicated fragment includes genes such as *OR11H2* and *OR4Q3*, and *OR4Q3* is associated with congenital ectodermal dysplasia syndrome.
14	46,XN,dup(17)(q21.31)	The duplicated fragment includes genes such as SOST and DUSP3, and SOST is associated with craniodiaphyseal dysplasia; Chromosome 17q21.31 duplication syndrome
15	47,XY,+15,del(16)(p11.2)	Chromosome 16p11.2 deletion syndrome
16	47,XX,+2,del(16)(p13.11)	16p13.11 recurrent microdeletion
17	46,XX/47,XX,+19,dup(16)(p13.3)	Chromosome 16p13.3 duplication syndrome
18	47,XN,+9,dup(16)(p11.2)	The duplicated fragment includes genes such as CD19 and ATXN2L. Cases with duplication in this area show mental retardation.

chromosomal abnormalities (*20*), which is consistent with our study. It is proved that aneuploidy of embryonic chromosome increases dramatically with increasing maternal age. Hassold *et al*. have reported that the incidence of trisomy is about 2% when mothers are in their 20s, while about 35% of women in their 40s are carrying babies with trisomies (*21*). Meanwhile, a study by Kroon *et al*. showed that the rate of embryonic aneuploidy from women aged over 35 was significantly higher than that in the group of ≤ 35 years old (45.7% *vs* 34.8%) (*22*). Our study also showed a sharp increase of trisomies when the maternal age exceeded 35 years

old. The existing evidence leads to the conclusion that advancing maternal age is associated with the increasing possibility of miscarriage. Over 90% of embryonic trisomies are caused by errors in paternal and maternal gametogenesis, and most of these errors occur in oocyte meiosis, while some others occur during the first few mitotic divisions of the fertilized ovum (*23,24*). A review by Chiang stated that the majority of abortus trisomies were maternal rather than paternal (*25-30*), and it was the aging oocyte rather than the uterus to blame (*31,32*).

In our study, among all the trisomies found in the abortuses, trisomy-16 is the most common type, which is in accordance with the study by Jia *et al*. (*33*). Further investigation into all these types of trisomies leads to an observation that trisomy-16 has hardly been found in live births, while fetuses with other types of trisomies may live with certain types of malfunctions (*34-37*). The exact mechanism of trisomy-16 being lethal is still unclear.

We employed NGS for karyotype analysis in this study. Compared with the traditional chromosome karyotyping method, NGS has its advantages in methodology, specifically in resolution and coverage. Our results have proved that NGS is both efficient and reliable; however, there are drawbacks to its use. The final result of NGS is formed by assembling various lengths of fragmented reads together with stringent algorithms applied to ensure the accuracy of the process. Therefore, one concern is false positive results, which are inevitable due to the large volume of acquired data. So NGS results should be interpreted in combination with other evidence. Another concern is that only part of the NGS result is of clinical significance.

Although abnormal karyotypes are confirmed in nearly half of the abortuses, the proportion of abnormal karyotypes in the parents is notably slight, at a percentage ranging from 2.78% to 4.32% in several retrospective studies (*38-40*). Miscarriages caused by abnormal karyotypes can occur in couples with normal chromosome karyotypes. The detective rate of abnormal karyotypes between villus of abortus and parental peripheral blood is significantly different, as is the abnormal karyotype profile. Studies have shown that abnormal chromosomal karyotypes in peripheral blood samples of RSA couples mainly involve balanced translocation, Robertsonian translocation, inversions, and X chromosome inactivation (*41*). Structural abnormalities with balanced translocations are the most common type of karyotype abnormalities detected in RSA couples, accounting for 3%-6% of the total abnormalities depending on the population studied (*42,43*). Incidence of reciprocal translocations is one in 500 live births, and the carriers may present normal physical and intellectual development. However, they are at risk for having genetically abnormal offspring because of the occurrence of unbalanced translocations in germ cells. Even if those germ cells with unbalanced translocations

are fertilized and developed into embryos, most of them end up with pregnancy loss. Preimplantation genetic diagnosis is now utilized to help couples with detected abnormal chromosomal karyotypes to analyze and select genetically healthy embryos before implantation, which greatly improves pregnancy outcomes. Robertsonian translocation occurs in about 1/1,000 of the general population (*44*). The RSA rate in couples with Robertsonian translocation is significantly higher (*45,46*). A study by Kolgeci revealed that Robertsonian translocation between 15q;15q resulted in intrauterine death and spontaneous failures of all pregnancies (*45*).

There are a few limitations in the current study. We only collected information of fetal chromosomal aberrations and maternal age; the recommended chromosome analysis of both parents in RSA cases has not been performed. In addition, the distributions of recognized RSA risk factors such as antiphospholipid syndrome (APS) and major uterine anomaly of the patients should be further studied. It is estimated that the percentage of truly unexplained causes of RSA is around 25% (*47*).

In summary, our study suggests that abnormal embryonic karyotype is a main factor in RSA. A well-structured prenatal diagnosis, both clinically and genetically, and preimplantation genetic diagnosis for couples with detected abnormal chromosomal karyotypes, along with healthy life styles may be beneficial to improve reproductive outcomes for RSA couples.

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Supplemental Data

Supplementary Table 1. Geographic distribution of the patients

Region	Normal karyotype ($n = 388$)		Abnormal karyotype ($n = 393$)	
	n	%	n	%
Metropolitan Shanghai	135	34.8%	265	67.4%
Zhejiang Province	23	6.0%	37	9.4%
Jiangsu Province	22	5.7%	37	9.4%
Fujian Province	4	1.0%	6	1.5%
Anhui Province	9	2.3%	6	1.5%
Hubei Province	3	0.8%	4	1.0%
Jiangxi Province	6	1.5%	3	0.8%
Henan Province	1	0.3%	3	0.8%
Shandong Province	2	0.5%	3	0.8%
Sichuan Province	1	0.3%	2	0.5%
Xinjiang Uygur Autonomous Region	0	0.0%	2	0.5%
Hebei Province	1	0.3%	1	0.3%
Gansu Province	0	0.0%	1	0.3%
Yunnan Province	0	0.0%	1	0.3%
Heilongjiang Province	1	0.3%	0	0.0%
Missing data	180	46.4%	22	5.6%