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Original article

Co-occurrences of polymorphic heterochromatin regions of chromosomes and effect on reproductive failure

Yasemin Karaca[1,*], Erhan Pariltay, Lamiya Mardan[2], Emin Karaca, Asude Durmaz, Burak Durmaz, Ayca Aykut, Haluk Akin, Ozgur Cogulu

Ege University, Faculty of Medicine, Department of Medical Genetics, Izmir, Turkey

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ABSTRACT

Although the polymorphic heterochromatin regions of chromosomes (heteromorphisms) have been extensively studied for their phenotypic effects on humans, co-occurrences of chromosome 1, 9, 16 and Y heteromorphisms and of acrocentric variants have never been studied on humans with an objective scoring system. Here we compared the frequencies of individual heteromorphisms on a total of 602, 768 and 224 patients with the indications of infertility, recurrent miscarriage and in vitro fertilization (IVF) failure, respectively and on 272 controls. Then we examined whether there were significant co-occurrences between heteromorphisms within and between the groups. There were no statistically significant differences in the frequencies of heteromorphisms between the groups. Both statistically significant and non-significant correlations were observed within the non-acrocentric and certain acrocentric heteromorphisms in each group. When these co-occurrences were examined between the groups, a 2.2 fold increased risk of IVF failure in males in the presence of either chromosome 13 or chromosome 21 variants was observed (95 %CI:1.1–4.2). We conclude that the simultaneous manifestations of heteromorphisms have no effect on reproductive failure. There seems to be a correlation between the non-acrocentric heteromorphisms (1qh+, 9qh+, 16qh + and Yqh+/-), which might be the result of complex interactions of formation of these heterochromatin regions. The correlations observed between certain acrocentric chromosomes might be related to satellite association and nucleolus formation. The increased risk observed in males with IVF failure in the presence of either chromosome 13 or 21 variants should be interpreted cautiously due to the heterogeneity of the group.

1. Introduction

Differences in the staining pattern and size of the constitutive heterochromatin regions of chromosomes between individuals is defined as heteromorphisms. These are most apparent in the pericentric regions of chromosome 1, 9 and 16; in the distal part of the long arm of the Y chromosome; short arms, stalks and satellites of the acrocentric chromosomes. Heteromorphisms have been considered as normal variations of normal karyotypes, since they are composed of tandemly organized, highly repetitive sequences of satellite DNA with apparently no protein coding potential [1,2]. Despite the acceptance of heteromorphisms as normal variants, there have been numerous studies with contradicting results regarding their clinical consequences, hence a great deal of controversy exists.

Although satellite DNA sequences that constitute the major part of the heterochromatin does not encode proteins, it does not necessarily mean that it's transcriptionally inactive or junk DNA. In fact, in 2012, ENCODE shared their results regarding the non-coding part of the genome and indicated that 80.4 % of the genome was involved in an RNA and/or chromatin related biochemical event [3]. It has been revealed that 62.1 % and 74.7 % of the genome overlapped with processed and primary transcripts, respectively [4]. Transcripts derived from alpha-satellite repeats in humans mediate localization of CENP-C and INCENP into the nucleolus in interphase and relocalization to the centromeres during mitosis [5]. It is indicated that a centromeric long noncoding RNA (lncRNA) is implicated in CENP-A loading to the centromere [6]. LncRNAs are also involved in heterochromatization of certain sequences, such as telomeric repeat containing RNA (TERRA) which is involved in the heterochromatization of telomeric chromatin [7]. Other than the aforementioned roles of the transcripts derived from

* Corresponding author at: Ankara University, Faculty of Medicine, Department of Medical Genetics, A. Adnan Saygun Street, Altindag, Ankara, Turkey.
E-mail address: ykaraca@ankara.edu.tr (Y. Karaca).
[1] Ankara University Faculty of Medicine Department of Medical Genetics, Ankara, Turkey.
[2] Adnan Menderes University Faculty of Medicine Deparment of Medical Genetics, Aydın, Turkey.

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centromeric and pericentromeric heterochromatin, maintenance and spreading of heterochromatin is also quite complex and invokes the question of whether heterochromatin dynamics include inter-chromosomal interactions as well [8,9].

In view of these findings, it is reasonable to continue examining the clinical and phenotypic effects of heteromorphisms. Therefore, we share our own clinical experience of heteromorphisms with patients who have been referred to our clinic for infertility, recurrent miscarriage (RM) and in vitro fertilization (IVF) failure indications. First, we screened and compared the frequencies of heteromorphisms in our patient cohort and a control group retrospectively. Secondly, we examined whether there were significant simultaneous manifestations of individual heteromorphisms, since the complex nature of heterochromatin dynamics might point to such co-occurrences which might have clinical consequences.

2. Materials and methods

We conducted a retrospective study on the karyotypes of patients who were referred to Ege University Medical Genetics Clinic for infertility, RM and IVF failure between January 2015 and April 2018. A total of 1866 individuals were included in the study. Written consent was obtained from all subjects.

2.1. Patient selection

A total of 301 couples with failure to achieve pregnancy for at least 12 months in spite of unprotected intercourse were included in the study. Medical history, investigations for ovarian functions and reserves, thyroid functions and anatomical features were normal for all females and all the cases have been considered as idiopathic. Physical examination, history and semen analysis were normal for their male partners and these cases have been considered as unexplained infertility as well. 384 couples with a history of two or more pregnancy losses before the 20th week of gestation were included in the study. Medical and obstetric history, genetic analysis of both partners, thyroid screening and anatomical investigations of female partners included in the group were all normal; thus all the cases were considered as unexplained RM. 112 couples with at least two failed IVF cycles constituted the IVF failure group. The total number of patients in the fertile group was 272 (136 couples). These control cases were patients who had undergone karyotype analysis from peripheral blood for parental analysis as a result of having a previous child with a genetic disease, a child with an abnormal genetic test result (microarray, conventional karyotype analysis), a fetus with an anomaly detected in the prenatal USG.

All the patients included in the study had normal chromosomes. Patients with karyotypes with numerical and structural chromosomal abnormalities, including mosaicism, and those with AZF deletions detected by Y microdeletion analysis were excluded from the study.

2.2. Karyotype analysis

Chromosome karyotype analysis was carried out on peripheral blood leukocytes. The standard laboratory protocol using GTG banding was performed on all samples. At least 20 metaphases and 5 karyotypes were analyzed using Cytovision 3.6 program at a band resolution of 450-550. The number of analyzed metaphases and karyotypes was increased as necessary. The analysis was carried out by three independent observers.

2.3. Classification of heteromorphisms

Heteromorphisms of pericentromeric regions of chromosome 1, 9 and 16 were designated as 1qh+, 9qh + and 16qh+, respectively. Inversion of chromosome 9 was represented as inv(9). Polymorphisms of the heterochromatin region of chromosome Y were designated as Yqh + or Yqh-. Polymorphisms of short arms, stalks and satellites of acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) were depicted as p+, pstk + and ps+, respectively.

Table 1
Size levels of heterochromatin regions.

Level 1	0.5 < x 16p	Very small
Level 2	≥ 0.5–1 × 16p	Small
Level 3	> 1-1.5 × 16p	Intermediate
Level 4	> 1.5-2 × 16p	Large
Level 5	> 2 × 16p	Very large

In order to evaluate the heteromorphisms of chromosomes 1, 9 and 16 objectively, a scoring system based on the comparison of each of them to the short arm of chromosome 16 (16p) was utilized which was developed by Verma et al., originally for C banding [10]. We also applied the same scoring system to the size differences of the heterochromatin segment of chromosome Y, instead of comparing the total length of the chromosome to the lengths of other certain chromosomes. The reason for this approach was the demonstration by Q-banding of the contribution of both the genetically active non-fluorescent segment (euchromatin) and the genetically inactive brilliant fluorescent segment (heterochromatin) to the total length variation of chromosome Y and we particularly aimed to evaluate the heterochromatin segment only [11].

The size of the heterochromatin regions of the chromosomes was enumerated between levels 1–5 (Table 1) according to their lengths compared to the length of 16p. The levels of chromosome 9 are demonstrated in Fig. 1 as an example to the method. The most frequent level was set as the threshold and the values above the threshold were accepted as heteromorphisms and designated as qh+. The value that was below the most frequent level was accepted as qh- for the Y chromosome. In the presence of inversion in one of the homologues, the size of the heterochromatin region of the other homologue was disregarded. For acrocentric chromosomes, the variant had to be at least twice the size of the corresponding region of its homologue to be accepted as a heteromorphism [12].

2.4. Statistical analysis

SPSS 25.0 (IBM Corporation, Armonk, New York, United States) software was utilized for statistical analysis. For the analysis of categorical variables, Pearson Chi-Square and Fisher Exact tests were executed by Monte Carlo stimulation technique. Column ratios were compared with each other and expressed as p value with the correction of Benjamini-Hochberg. Categorical variables were demonstrated as n (%) and values below $p = 0.05$ were accepted as statistically significant.

2.5. Ethical approval

This study was approved by the Ethics Committee of Ege University Hospital on 20/03/2019 (Reference number: 19-3.1 T/43).

3. Results

3.1. Prevalence of heteromorphisms

The size levels of heterochromatin regions of chromosomes 1, 9, 16 and Y in the total population are shown in Table 2. For chromosomes 1 and 9, the most frequent size levels were level 1 and level 2. Therefore level 3 and higher values were accepted as heteromorphisms, 1qh + and 9qh+, respectively. For chromosome 16, the most frequent size level was level 1, hence level 2 and higher values were accepted as 16qh+. For the Y chromosome, the most frequent size level was level 2.

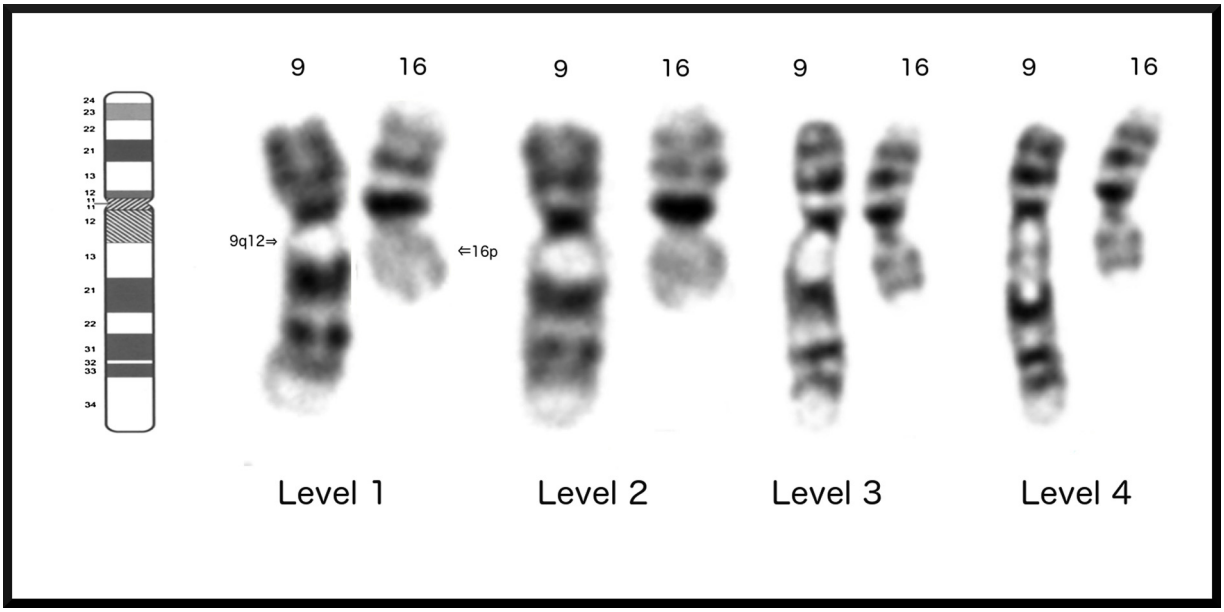

Fig. 1. The levels of chromosome 9 heteromorphism according to the scoring system adapted from Verma et al. In this classification of heteromorphisms, heteromorphic region of the chromosome 9 (9q12) is compared to the short arm of chromosome 16 (16p) and scored accordingly (see Table 1). For the purpose of demonstration, chromosome 16 is shown upside down next to each 9q12 region. On the far left, an ideogram of chromosome 9 is shown.

Table 2
Distribution of Different Sized Polymorphisms.

	Number of Homologue Chromosomes			
Size Levels	1	9	16	Y
Very small (1)	1426	1705	3415	171
Small (2)	1974	1722	308	584
Intermediate (3)	290	208	9	151
Large (4)	40	16		27
Very Large (5)	2	1		
Total	3732	3652[a]	3732	933

[a]Excluding the inv(9) carrying homologue sets.

Therefore level 1 was accepted as Yqh-, and level 3 and 4 were accepted as Yqh+. The distribution of determined heteromorphims is shown in Table 3.

The most common heteromorphisms in females were chromosome 21 variants (17.9 %), 1qh+ (14.7 %), 1qh+ (15.9 %) and 1qh+ (14.1 %) in IVF failure, infertility, RM and control groups, respectively. In males, the most common heteromorphisms were Yqh- (19.6 %), Yqh+ (22.3 %), Yqh- (19.5 %) and Yqh+ (19.9 %) in IVF failure, infertility, RM and control groups, respectively. There were no significant differences between the indication groups and the control group or sexes.

Among the D/G group chromosomes, the most frequent chromosome with heteromorphic changes was chromosome 21 in females within all the groups, with prevalence of 17.9 %, 12.6 %, 10.4 % and 12.5 % in IVF failure, infertility, RM and control groups, respectively. There were no significant differences. In males, the most heteromorphic acrocentric chromosome was chromosome 22 in IVF failure (16.1 %), chromosome 21 in infertility (11 %), chromosome 15 in RM (11.5 %), chromosome 22 in the control group (12.5 %), with no significant differences. The most common heteromorphic variant observed in all acrocentric chromosomes was pstk+.

3.2. Simultaneous manifestations of heteromorphisms and their significance

To investigate the simultaneous manifestations of heteromorphisms, we first examined whether there was a meaningful increase or a decrease in the frequency of a variant in the presence of another within each group by pair-wise comparison. In general, for each group, there was a tendency to increase in the frequencies of the non-acrocentric heterochromatic variants (1qh+, 9qh+, 16qh + and Yqh+) in the presence of each other and also a decrease in the frequencies of 1qh+, 9qh + and 16qh + in the presence of Yqh-. A similar positive correlation observed in all groups between chromosomes 13 and 21 (except in the IVF failure group), chromosomes 14 and 21, chromosomes 15 and 21, chromosomes 15 and 22 and chromosomes 21 and 22 (except the females in the control group) in the acrocentric chromosome variants. It was not possible to generalize the correlations found between non-acrocentric and acrocentric chromosomal variants.

Some of the correlations were significant in certain groups. There were significant positive correlations between 1qh + and 16qh + in males with infertility, females with RM and males with IVF failure; between 1qh + and Yqh + in IVF failure and RM groups (p values 0.039, 0.049 and 0.03, 0.002 and 0.005). The negative correlation between 1qh + and Yqh- in IVF failure ($p = 0.002$) and both the positive and negative correlations between 16qh+ and Yqh+/Yqh-, respectively ($p = 0.01$) were also statistically significant. Within the acrocentric groups, increases in the frequencies of chromosome 15 and chromosome 22 variants in the presence of each other were significant in males in the RM group, males and females in the infertility group and males in the control group (p values 0.027, 0.008, 0.004 and 0.017, respectively). The similar correlations were also observed between chromosome 13 and 21 variants in males with RM ($p = 0.000$) and between chromosome 13 and 22 variants in males with infertility ($p = 0.038$).

Although non-significant statistically, the relationship of chromosomes 21 and 22 variants with each other had a distinctive pattern in females. The frequencies of chromosome 21 and 22 variants were decreased in the presence of each other in the control group, but increased in other females, with a statistical significance in the RM group ($p = 0.026$).

Secondly, we investigated whether there was an association of the simultaneous manifestations of chromosomal variants with the phenotypes of reproductive failure. When the combination analysis was performed, it was found that the presence of either chromosome 13 or chromosome 21 variants was significantly higher in IVF failure in males and in total compared to the control group, with p values of 0.040 and

Table 3
Frequencies of heteromorphisms.

	Female				P	Male				P
	IVF failure n (%)	Infertility n (%)	Recurrent miscarriage n (%)	Control n (%)		IVF failure n (%)	Infertility n (%)	Recurrent miscarriage n (%)	Control n (%)	
1qh+										
Homozygous -	95 (84.8)	257 (85.4)	323 (84.1)	117 (86.0)	**0.989** [P]	94 (83.9)	248 (82.4)	327 (85.2)	120 (88.2)	**0.604** [F]
Heterozygote +	14 (12.5)	36 (12.0)	53 (13.8)	16 (11.8)		16 (14.3)	42 (14.0)	46 (12.0)	15 (11.0)	
Homozygous +	3 (2.7)	8 (2.7)	8 (2.1)	3 (2.2)		2 (1.8)	11 (3.7)	11 (2.9)	1 (0.7)	
9qh+/inv(9)										
Homozygous -	98 (87.5)	267 (88.7)	335 (87.2)	117 (86.0)	**0.801** [F]	97 (86.6)	268 (89.0)	329 (85.7)	121 (89.0)	**0.864** [F]
Heterozygote +	10 (8.9)	24 (8.0)	32 (8.3)	12 (8.8)		12 (10.7)	24 (8.0)	38 (9.9)	11 (8.1)	
Homozygous +	3 (2.7)	3 (1.0)	11 (2.9)	3 (2.2)		0 (0.0)	4 (1.3)	5 (1.3)	2 (1.5)	
inv(9)	1 (0.9)	7 (2.3)	6 (1.6)	4 (2.9)		3 (2.7)	5 (1.7)	12 (3.1)	2 (1.5)	
16qh+										
Homozygous -	97 (86.6)	259 (86.0)	328 (85.4)	118 (86.8)	**0.890** [P]	95 (84.8)	262 (87.0)	327 (85.2)	117 (86.0)	**0.944** [F]
Heterozygote +	14 (12.5)	33 (11.0)	46 (12.0)	16 (11.8)		12 (10.7)	28 (9.3)	45 (11.7)	15 (11.0)	
Homozygous +	1 (0.9)	9 (3.0)	10 (2.6)	2 (1.5)		5 (4.5)	11 (3.7)	12 (3.1)	4 (2.9)	
Yqh+/Yqh-										
-	–	–	–	–		22 (19.6)	49 (16.3)	75 (19.5)	25 (18.4)	**0.671** [P]
+	–	–	–	–		19 (17.0)	67 (22.3)	65 (16.9)	27 (19.9)	
N	–	–	–	–		71 (63.4)	185 (61.5)	244 (63.5)	84 (61.8)	
Chromosome 13 variants										
Absent	105 (93.8)	283 (94.0)	362 (94.3)	126 (92.6)	**0.930** [P]	98 (87.5)	275 (91.4)	349 (90.9)	124 (91.2)	**0.678** [P]
Present	7 (6.3)	18 (6.0)	22 (5.7)	10 (7.4)		14 (12.5)	26 (8.6)	35 (9.1)	12 (8.8)	
Chromosome 14 variants										
Absent	101 (90.2)	269 (89.4)	349 (90.9)	125 (91.9)	**0.839** [P]	104 (92.9)	277 (92.0)	362 (94.3)	124 (91.2)	**0.556** [P]
Present	11 (9.8)	32 (10.6)	35 (9.1)	11 (8.1)		8 (7.1)	24 (8.0)	22 (5.7)	12 (8.8)	
Chromosome 15 variants										
Absent	103 (92.0)	274 (91.0)	349 (90.9)	130 (95.6)	**0.366** [P]	98 (87.5)	276 (91.7)	340 (88.5)	122 (89.7)	**0.502** [P]
Present	9 (8.0)	27 (9.0)	35 (9.1)	6 (4.4)		14 (12.5)	25 (8.3)	44 (11.5)	14 (10.3)	
Chromosome 21 variants										
Absent	92 (82.1)	263 (87.4)	344 (89.6)	119 (87.5)	**0.218** [P]	96 (85.7)	268 (89.0)	341 (88.8)	124 (91.2)	**0.603** [P]
Present	20 (17.9)	38 (12.6)	40 (10.4)	17 (12.5)		16 (14.3)	33 (11.0)	43 (11.2)	12 (8.8)	
Chromosome 22 variants										
Absent	100 (89.3)	268 (89.0)	354 (92.2)	125 (91.9)	**0.478** [P]	94 (83.9)	270 (89.7)	346 (90.1)	119 (87.5)	**0.287** [P]
Present	12 (10.7)	33 (11.0)	30 (7.8)	11 (8.1)		18 (16.1)	31 (10.3)	38 (9.9)	17 (12.5)	

[P] Pearson Chi-Square Test (Monte Carlo), [F] Fisher Freeman Halton Test(Monte Carlo); Post Hoc Test: Benjamini-Hochberg correction, [A] Significant for IVF Failure group, [B] Significant for Infertility group, [C] Significant for Control group, [D] Significant for Recurrent miscarriage group.

0.021, respectively (Supplementary Table I). The increase in the risk of IVF failure in males in the presence of either of these variants was estimated to be 2.2 fold (95 %CI:1.1–4.2). In IVF failure, although without statistical significance, the frequencies of chromosome 13 and 21 variants had been decreased in the presence of each other, contrary to the rest of the groups.

4. Discussion

We utilized a scoring system that was developed by Verma et al., originally for C-banding to evaluate the size differences of heterochromatin regions of chromosomes 1, 9 and 16. They had found the most frequent levels to be level 3 for chromosome 1, level 2 for chromosome 9 and level 1 for chromosome 16. Our findings are similar except for chromosome 1, for which the most frequent level was level 2 in our study. These results indicate that GTG banding could be an adequate technique to evaluate the heterochromatin regions of chromosomes, although it cannot replace C-banding for this purpose. We believe such a scoring method for the objective evaluation of heteromorphisms is essential for reproducibility and also for the inclusion of the heteromorphisms that may be missed out by other classifications.

We applied the same scoring system to the heterochromatin region of Y chromosome as well instead of the traditional evaluation of chromosome Y variants, which utilizes the comparison of its length to other certain chromosomes'. It is known that both heterochromatin and euchromatin segments contribute to the length of chromosome Y and in order to examine the heterochromatin segment in particular, such adaptation was needed in our opinion.

The most common heteromorphism in females was 1qh + in the RM, infertility and control groups. In females from the IVF failure group, chromosome 21 variants in total were higher than 1qh+. The most common heteromorphism in males was Yqh + in the control and infertility groups and Yqh- in RM and IVF failure. Due to the utilization of different scoring systems to define heteromorphisms and lack of uniformity in the classification of variants between the studies, it is difficult to compare our data with the relevant literature. On the other hand, the identification of inv(9) in a standard GTG banding is fairly objective and the incidence of inv(9) in our cohort is similar to those observed in other studies and in general population.

Studies with contradicting results exist regarding the causal role of heteromorphisms in infertility. Madon et al., Minocherhomji et al., Sahin et al., Mierla and Stoian and Yakin et al. are among those researchers who have found significant associations between heteromorphisms and infertility [12–16]. Kalantari et al. on the other hand, found no association between Y chromosome variants with sperm counts or male infertility [17]. Dong et al. included 1751 males and 1424 couples with reproductive failure and 777 fertile control individuals in their study and found no significant differences in the frequencies of heteromorphisms [18]. Moreover, they also examined the karyotypes of family members of 38 heteromorphic probands and the same heteromorphic karyotypes of the probands were also observed in other family members with no reproductive failure. Concordantly with their findings, we did not find any significant differences in the frequencies of heteromorphisms between the infertility and control groups in the present study.

The role of heteromorphisms in RM is also quite controversial. Genest, Patil and Lubs, Wang et al. have found significant associations between Y chromosome polymorphisms and spontaneous abortions

[19–21]. Hemming and Burns, Blumberg et al., Nie and Lu on the other hand, indicated there was no association between heteromorphisms and RM [22–24]. The findings of the present study support the absence of an association between heteromorphisms and RM.

Studies examining the relationship of heteromorphisms with IVF failure are relatively scarce. Liang et al. observed a negative effect of male polymorphisms on fertilization rates and Xiao Z et al. determined that the outcomes of IVF treatment were significantly worse in Yqh + carrying couples [25,26]. However, Hong et al. indicated that there was no effect of polymorphic variants on the outcome of in vitro fertilization [27]. In support of their findings, we did not find any significant associations between heteromorphisms and IVF failure in the present study.

When we investigated the simultaneous manifestations of heteromorphisms in individual groups without making a comparison between the groups, we observed many potentially important correlations between the non-acrocentric heteromorphisms (1qh, 9qh, 16qh, Yqh). While some of these were statistically significant in certain groups, they were also present for the rest of the groups without statistical significance. We believe that the value of p is not a reliable determiner in this analysis due to the low number of cases with positive variants. Nevertheless, this observed general trend between the non-acrocentric heteromorphisms, together with the increasing knowledge of heterochromatin and its formation makes it conceivable that the heterochromatization of these chromosomes might be interrelated to each other. If there is such a process indeed, it does not seem to have an effect on the phenotypes examined in this study.

For the acrocentric variants, we observed positive correlations between chromosomes 13 and 21 (except for the males of IVF failure group), 14 and 21, 15 and 21, 15 and 22, and also 21 and 22 (except for the females of the control group) with statistical significance in several groups. As mentioned above, statistical analysis is not reliable here as well, due to low number of cases with positive variants. If indeed these correlations hold true, underlying mechanism could be explained by below-mentioned phenomenon of the satellite association. The satellite association could also account for the contradictory correlations of chromosomes 21 and 22 between the control group and the others in females.

In metaphase preparation of human chromosomes, acrocentric chromosomes may be observed in relatively close proximity to each other more often than expected and this phenomenon has been referred to as satellite association. Satellite association probably plays a role in the nucleolus formation and has been suggested to be involved in the etiology of some structural and numerical chromosomal abnormalities [28]. Jacobs et al. indicated chromosome 21 to be the most frequently involved acrocentric chromosome in satellite associations, followed by chromosome 22 [29]. Cohen and Shaw and Van Brink et al. also observed the G group chromosomes (chromosomes 21 and 22) to be involved in satellite associations more frequently than the D group chromosomes (chromosomes 13, 14 and 15) [30,31]. The patterns we observed between the acrocentric chromosomes in this study, whether they are statistically significant or not, might be supportive of this phenomenon. The association of chromosome 21 and 22 in particular, should be elucidated by further studies with larger sample sizes.

When we compared the simultaneous manifestations of all variants, we found a 2.2 fold increased risk of IVF failure in males in the presence of either chromosome 13 or chromosome 21 variants (95 %CI:1.1–4.2). Due to the low number of cases, this finding should be approached skeptically in terms of statistical significance and the heterogeneity of the group should be considered. In our opinion, except for the association of chromosomes 21 and 22 with each other in females, the other acrocentric variant correlations are probably indicative of a physiological process and not related to the phenotypes investigated in this study.

The major strength of the present study is the utilization of a scoring system that has been developed previously. The criteria of “twice the size of its homologue” which has been the most extensively used determination of a variant misses out many true variants in our opinion. Adaptation of the scoring system to include the Y chromosome heterochromatin is a novelty of our study to our knowledge. Our effort to examine the impact of simultaneous manifestations of heteromorphisms on reproductive failure and the associations of these variants with each other is a new approach to the studies of heteromorphisms.

There are several limitations to this present study. We were unable to confirm the heteromorphisms observed by GTG banding with C- and NOR-banding. Since FISH and microarray analyses are not routinely performed for infertility, RM and IVF failure, we cannot exclude cryptic translocations, microdeletions and microduplications. We did not have detailed information regarding the indication, the process and the criteria of failure of IVF treatment in patients with IVF failure. The cases included in the control group, although were proven to be fertile, mostly had a history of a previous abnormal offspring. Some cases were pregnant couples with an abnormal ultrasound finding and although no chromosomal anomaly indicating a termination had been detected in these fetuses, we do not have information regarding the health of children born. The sample size was insufficient for certain analyses as mentioned above and future studies with larger sample sizes and more homogenous groups using the same scoring system is warranted. We were unable to confirm the heritability of these heteromorphisms and the significance of the co-occurrences of them in other family members, hence studies examining family members as well as probands are needed. Future studies involving products of conception, malformed fetus/baby/sperms/spare oocytes and embryos may prove significant.

Author contribution

YK, EP and OC contributed to the design of the study. YK, LM and EP performed the research. BD, AD and EK analyzed the data. YK and EP wrote the paper. AA, OC and HA revised the manuscript. All authors read and approved the final version of the manuscript.

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Declaration of Competing Interest

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