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Bridging the Gap between AZF Microdeletions and Karyotype: Twelve Years' Experience of an Infertility Center

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Purpose: Despite all past efforts, the current guidelines are not explicit enough regarding the indications for performing azoospermia factor (AZF) screening and karyotype, burdening clinicians with the decision to assess whether such tests are meaningful for the infertile male patient. These assessments can be costly and it is up to the healthcare practitioner to decide which are necessary and to weigh the benefits against economic/psychological harm. The aim of this study is to address such gaps and provide update on current management options for this group of patients.

Materials and Methods: To address such gaps in male infertility management and to elucidate whether AZF screening is indicated in individuals who concomitantly harbor chromosomal abnormalities we conducted a retrospective cohort analysis of 10,388 consecutive patients with non-obstructive azoospermia (NOA) and severe oligozoospermia.

Results: Previously, it has been suggested that all NOA cases with chromosomal defects, except males with 46,XY/45,X karyotype, have no indication for AZF screening. Our findings revealed that cases carrying the following chromosomal abnormalities inv(Y)(p11.2q12); idic(Y)(q11.2); 46,XY,r(Y); idic(Y)(p11.2) and der(Y;Autosome) (76/169; 44.9%; 95% CI, 37.7–52.5) should also be referred for AZF deletion screening. Here, we also report the correlation between sperm count and AZF deletions as a secondary outcome. In accordance with previously reported data from North America and Europe, our data revealed that only 1% of cases with $>1\times10^6$ sperm/mL had Y chromosome microdeletions (YCMs).

Conclusions: In the era of assisted reproduction, finding cost-minimization strategies in infertility clinics without affecting the quality of diagnosis is becoming one of the top prioritized topics for future research. From a diagnostic viewpoint, the results reflect a need to reconsider the different karyotype presentations and the sperm count thresholds in male infertility guidelines as indicators for YCM screening during an infertility evaluation.

Keywords: Azoospermia; Gene deletion; Karyotype; Klinefelter syndrome; Mosaicism; Y chromosome microdeletions

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INTRODUCTION

Infertility globally prevails at a percentage of approximately 15%, where nearly half of these cases are attributed to male factor infertility [1]. From an etiological perspective, male infertility is a multifactorial condition, in which genetic factors are estimated to account for 15% of cases, affecting a wide spectrum of physiological processes involved in fertility, from hormonal homeostasis to spermatogenesis, in both quantitative and qualitative terms [2]. Therefore, genetic testing for patients with impaired reproductive dynamics is of crucial importance not only for its particular preventive value in reducing the risk of transmission of genetic conditions to progeny through assisted reproductive techniques, but also for shedding light on genetic defects that may adversely affect the chance of sperm retrieval through surgical extraction [1]. Karyotyping and sequence tag site (STS)-multiplex polymerase chain reaction (PCR) analysis of Y chromosome microdeletions (YCMs) are genetic tests commonly requested by clinicians for patients with a sperm concentration $<5\times10^6$/mL as a part of the infertility workup prior to assisted reproductive technology (ART) [2,3]. Although, such defects are frequently validated in patients with azoospermia (1 in 4 cases), the list of potential genetic causes underlying male infertility is constantly expanding [2].

The human Y chromosome accounts for 2% to 3% of the estimated haploid genome size (~60 Mb) and conceals the genes encoding for the proteins which are not only involved in testicular development but also in triggering and maintaining normal spermatogenesis [4]. A unique feature of the human Y chromosome is the presence of inverted duplicates with a high-degree of sequence identity, also called palindromes, on the long arm of the chromosome (Yq11). This peculiarity facilitates the process of gene conversion to modulate genetic diversity in equilibrium, while maintaining the structural integrity of the genome [3]. Nevertheless, it has the potential to predispose the region to intra-chromosomal loss of genetic material during meiosis, causing a spectrum of outcomes ranging from common polymorphisms to pathological alterations in a set of genes involved in spermatogenesis and male infertility [5]. The region in the distal portion of Yq11 called azoospermia factor (AZF) was the first to be reported as a potential locus to undergo deletions in an early study that confirmed this finding in six azoospermic males [6]. Indeed, YCMs are estimated to be the cause of severe spermatogenesis failure in 1:12 infertile patients worldwide. The incidence in the general population is around 1:4,000 men [1].

Regarding molecular analysis of the Y chromosome in patients harboring chromosomal aberrations, two issues should be considered. First, the result of an interim international survey regarding the cost reduction needed in infertility treatments signified that omitting unnecessary testing may alleviate the costs even though it is currently ambiguous which tests to abandon and how the necessity or the value of each test is determined. This is one of top 10 prioritized topics for future research in the era of assisted reproduction [7]. Second, by reviewing the literature, it seems there are still uncertainties about which patients have an indication for YCM screening during the diagnostic workup. Increasing evidence suggests a need for larger studies to fill the gap between basic research and clinical practice in order to allow the development of a standard framework for guiding healthcare professionals [8-11].

The current retrospective study aims to investigate the association between YCMs and karyotype status by recruiting the largest cohort of patients with primary infertility to date, to provide the required insight of the karyotype-genotype-phenotype correlations towards establishing a framework for future guidelines to meet the demands of modern male infertility personalized management.

MATERIALS AND METHODS

1. Ethics statement

This research work has been approved by the Royan Reproductive Biomedicine Research Center Ethics Committee (approval number: IR.ACECR.ROYAN.REC.1399.117) on February 27, 2021. The study involved the extended analysis of an existing database, without any patient identifiers linking individuals to the data. During admission, a written informed consent was obtained from the patients for the use of their anonymous data for academic/research purposes.

2. Study design, duration, and population

This retrospective cohort analysis included 10,388 cases of male infertility due to non-obstructive azoospermia (NOA) (60.7%; 95% confidence interval [CI], 59.7–61.7) or severe oligozoospermia (SO) ($<5\times10^6$ sperm/

mL) (39.3%; 95% CI, 38.3–40.3), that were referred to the medical genetics lab of a tertiary referral center (Royan Infertility Clinic) for karyotyping and YCM assessment over the last 12 years (March/2009–February/2021). The mean age of the participants was 33.4±6.1 years (range 20–46 y). Patients with testicular/anatomical anomalies (*e.g.*, bilateral undescended testis, or torsion), urogenital infection/inflammation, hypogonadotropic hypogonadism, and history of viral orchitis, anabolic steroid abuse, testosterone replacement therapy, chemo-radiotherapy during the past 12 months were excluded from the study.

3. Detection of YCMs

For extraction of genomic DNA from peripheral blood, PAXgene® Blood DNA kit (Cat No.761133; Qiagen Inc., Hilden, Germany) was used. The procedure was conducted according to the instructions provided by the manufacturer. Extracted DNA was stored at -20°C. A total of six STS markers spanning the AZF region of chromosome Y were used for the detection of microdeletions in accordance with the European Academy Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) of laboratory recommendations [12], including: sY84 and sY86 (AZFa), sY127 and sY134 (AZFb) and sY254 and sY255 (AZFc). The *SRY* (sex-determining region of the Y) and *ZFX/ZFY* (zinc finger transcription factor) were optioned as internal PCR controls for Y and X/Y chromosome detection, respectively. To check reagent contamination, a blank no-template sample was also included in the analysis. The employed PCR condition has been discussed in detail, elsewhere [13].

Due to the increased time-span of the study and as normally anticipated following technological developments, the screening protocol for YCMs was altered with respect to the number of analyzed STSs. Before 2013, we applied a set of 8 STS markers sY83, sY84 (AZFa), sY134, sY142 (AZFb), sY154, sY157, sY158, sY254 (AZFc) and after 2013 the protocol shifted to comply with the suggested guidelines of EAA/EMQN [12], where sY84, sY134, and sY254 are in common with the previous protocol. Statistical analyses in the detection rates of the employed protocols implemented in our clinical practice throughout the study conduction period demonstrated no differences (95% CI, -0.0936 to 0.1475; p=0.8637), thus no alterations in the number of detected cases that could be attributed to the methodology modifications/microdeletion panels used.

4. Cytogenetic analysis

Cytogenetic assessment was conducted on G-banded metaphases, obtained after 72 hours of peripheral blood cell culture according to standard procedures. We utilized additional C- or Ag-NOR's banding techniques when necessary [14]. For each case, a minimum of five metaphases were fully analyzed, band by band, and 10 metaphase spreads were screened to exclude aneuploidies. In cases where chromosomal mosaicism was suspected, at least 50 metaphases were analyzed to exclude 10% mosaicism with 95% confidence level. The karyotypes were described in conformity with the current International System for Human Cytogenetic Nomenclature (ISCN) [15].

5. Semen analysis

Semen samples were obtained through ejaculation conforming to an abstinence period of 2–7 days. Semen analysis was conducted in fresh specimens for all recruited cases using computer assisted sperm analysis (CASA) according to the World Health Organization (WHO) recommendations and standards for the examination and processing of human semen [16,17] in terms of specimen characteristics, sperm concentration, morphology and motility.

6. Descriptive ANALYSIS

The MEDLINE database along with online life science journals and books were searched through PubMed by the algorithm (Klinefelter or KS or 47,XXY [Title/Abstract]) AND (AZF or Azoospermi* and/or microdeletion* [Title/Abstract]). The aim was to obtain available literature evidence on the frequency of YCMs (Table 1) and on the applied STSs for screening (Supplement Table 1) among azoospermic subjects with Klinefelter syndrome (KS; XXY). A conclusive search algorithm was formulated to allow adequate screening and included published reports until January 2022.

7. Statistical analysis

Results are reported as mean±standard deviation or percentages. The association between categorical variables was evaluated by chi-square or Fisher's exact test through the software GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA). Wilson score method was applied to estimate the difference between

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Table 1. Summary of literature data on AZF microdeletion frequency in patients with NOA and KS

#	Reference	No. of KS patients		Rate of classic AZF microdeletions, n (%)		No. of used markers	Ethnic origin
		Mosaic	Classic	KS	NOA		
1	[25]	1	2	1/3 (33.3)[a]	8/45 (17.8)	12	Spanish
2	[26]	4	11	0	12/168 (7.1)	35	American
3	[27]	0	21	0	NA	32	Japanese
4	[28]	0	6	0	1/9 (11.1)	60	Korean
5	[30]	5	NA	1/5 (20.0)	8/92 (8.7)	58	Slovenian
6	[29]	4	10	4/14 (28.6)[b]	NA	16	Indian
7	[11]	4	91	0	NA	5	Korean
8	[31]	0	1	0	26/49 (53.1)	34	Iranian
9	[32]	0	7	0	1/73 (1.4)	15	Turkish
10	[33]	0	208	1/208 (0.5)	39/3,179 (1.2)	15	German[d]
11	[20]	3	6	6/9 (66.7)	1/18 (5.5)	19	Tunisian
12	[10]	0	14	5/14 (35.7)	NA	9	Turkish
13	[34]	1	15	0	9/126 (7.1)	7	Kuwaiti
14	[35]	0	12	0	8/226 (3.5)	6	Slovak
15	[9]	2	78	0	NA	9	Chinese
16	[36]	5	11	1/16 (6.3)[c]	45/146 (30.8)	28	Syrian
17	[23]	0	111	28/111 (25.2)	NA	25	Chinese
18	[8]	4	92	0	67/556 (12.1)	16	Korean
19	[37]	0	191	0	35/547 (6.4)	14	Turkish[e]
20	[38]	2	116	1/118 (0.8)	46/429 (10.7)	9	Italian

AZF: azoospermia factor, KS: Klinefelter syndrome, NA: not available, NOA: non-obstructive azoospermia.
[a]47,XXY[2]/46,XY[48]. [b]Three cases with 47,XXY/46,XY and 1 case with 46,XY/47,XXY/48,XXXY/48,XXYY chromosomal pattern. [c]45,X/46,X,idic(Y)/47,XX,+idic(Y). [d]77% of cases were German; ethnicity background of the rest were Turkish, Russian/Slavic, Italian, Hispanic and Polish. [e]Mostly from Central Anatolia.

binomial proportions with 95% CI; which is the range of values we expect our estimate to fall between if we repeat the test, within a certain level of confidence (here 95%). All the statistical tests were two-tailed at a significance level of 0.05.

RESULTS

For a conclusive data presentation, and based on individual genetic background, the cohort was categorized as follows: (1) total cohort; (2) AZF-deleted patients; (3) males with both YCMs and an abnormal karyotype; (4) males with abnormal karyotype and without YCMs; and (5) patients lacking chromosomal aberrations or YCMs.

1. Analysis of the total cohort

The overall prevalence of YCM among the cohort was about 1:18 (578/10,388; 5.6%; 95% CI, 5.1–6.0). In the YCM positive population, over three-quarters had NOA (452/578; 78.2%; 95% CI, 74.6–81.4) and the remaining (126/578; 21.8%; 95% CI, 18.6–25.4) were males with SO (18.3% with <0.5 million sperm/mL, 2.2% 0.5–1 million sperm/mL and 1.2% 1–5 million sperm/mL). Accordingly, AZFc (332/578; 57.4%; 95% CI, 53.4–61.4) and AZFbc (124/578; 21.5%; 95% CI, 18.3–25.0) were the most frequent, followed by AZFabc (61/578; 10.6%; 95% CI, 8.3–13.3), AZFb (45/578; 7.8%; 95% CI, 5.9–10.3), and AZFa (16/578; 2.8%; 95% CI, 1.7–4.5). Among the severe oligozoospermic cohort, the majority of YCMs were diagnosed in those with sperm concentration of $<0.5\times10^6$ sperm/mL (106/126; 84.1%; 95% CI, 76.8–89.9) (Table 2). From the total of 10,388 cases with YCM screening, 97.6% (10,137/10,388) were karyotyped; thus the data belonging to the remaining 2.4% of cohort was excluded from the rest of the study (Table 3).

2. AZF-deleted patients

Of 565 total cases with YCMs, two-thirds presented with a normal karyotype (386/565; 68.3%; 95% CI, 64.4–

Table 2. Prevalence of complete YCM vis-à-vis sperm count

Spermogram	Of 578 cases with YCM		Proportion of YCMs									
			AZFa		AZFb		AZFc		AZFbc		AZFabc	
	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI
Azoospermic	452 (78.2)	74.65–81.37	16 (2.77)	1.71–4.45	44 (7.6)	5.72–10.07	208 (36)	32.18–39.98	123 (21.28)	18.14–24.8	61 (10.55)	8.3–13.32
Severe oligozoospermic (million/mL)												
<0.5	106 (18.34)	15.4–21.7	-		1 (0.17)	0.03–0.97	104 (18)	15.08–21.33	1 (0.17)	0.03–0.97		-
0.5–1	13 (2.25)	1.32–3.81	-			-	13 (2.25)	1.32–3.81			-	-
1–5	7 (1.2)	0.59–2.48	-			-	7 (1.2)	0.59–2.48			-	-

CI: confidence interval, YCM: Y chromosome microdeletion.

72.0). The prevalence of YCMs in subjects with normal karyotype was estimated as 4.6% (386/8,317); AZFc, AZFb, and AZFbc (78.5%, 10.9%, and 6.5%, respectively) had the highest prevalence rate. The prevalence in cases with cytogenetic heteromorphisms (normal variants) was decreased to 3.2% (10/313; 95% CI, 1.74–5.8).

3. Males with both YCMs and an aberrant karyotype

One-third of cases with YCMs concomitantly had an abnormal karyotype (excluding those with normal heteromorphisms) (169/565; 29.9%; 95% CI, 26.3–33.8) (Table 3). With respect to the frequency of YCMs, there was heterogeneity in this subgroup. Whereas some chromosomal aberrations including: 46,XX (in pure or mosaic constitution) (42/169; 24.9%; 95% CI, 19.0–31.9); 46,X,add(X) (3/169; 1.8%; 95% CI, 0.6–5.1); 45,X and its mosaic forms: 45,X/46,X,inv(Y); 45,X/46,X,del(Y); 45,X,der(Y;Autosome); 45,X/46,XY/46,X,idic(Y)(p11.2); 45,X/46,X,del(Y)/46,XY,r(Y); and 45,X/46,X,idic(Y)(q11.2) (19/169; 11.2%; 95% CI, 7.3–16.9); and inv(Y)(p11.2q12) (2/169; 1.2%; 95% CI, 0.3–4.2) always concurred with YCMs, which in sum, accounts for 39.1% (66/169; 95% CI, 32.0–46.6) of all chromosomal aberrations coexisting with YCMs, the other types, *e.g.*, 45,X/46,XY or 46,X,del(Y), sometimes concurred with YCMs and these comprised the rest of the subjects in this subgroup (60.9%, 103/169).

4. Cases with abnormal karyotype and without YCMs

In contrast to the above-mentioned subgroup, a set of chromosomal defects were exclusively observed in patients without YCMs, including Robertsonian translocations, autosomal inversions, autosomal translocations, and both mosaic and non-mosaic forms of Klinefelter (47,XXY) and non-mosaic forms of Jacobs (47,XYY) syndromes (Table 3). KS was determined in 10.2% (1,039/10,137; 95% CI, 9.7–10.9) amongst the cohort and was the most common genetic defect, with an added 9.1% (95/1,039; 95% CI, 7.5–11.0) of cytogenetically mosaic individuals. This subgroup comprised more than two-thirds of all cases with chromosomal aberrations (including heteromorphisms) (1,246/1,814; 68.7%; 95% CI, 66.5–70.8).

Table 3. Chromosomal status among the cohort with/without AZF deletion

Chromosomal status	Karyotype	Without YCMs	With YCMs						Total
			AZFa	AZFb	AZFc	AZFbc	AZFabc	All types of YCMs	
Total		9,810	16	45	332	124	61	578	10,388
Karyotyped		9,572	16	44	326	120	59	565	10,137
Normal	46,XY	7,931	16	42	303	25	0	386	8,317
Heteromorphism	ps+/pstk+	44	0	0	1	0	0	1	45
	46,XY,qh+	98	0	0	4	1	0	5	103
	inv(1)	12	0	0	0	0	0	0	12
	inv(2)	4	0	0	0	0	0	0	4
	inv(3)	24	0	0	1	0	0	1	25
	inv(9)	106	0	0	2	0	0	2	108
	inv(16)	0	0	0	1	0	0	1	1
	inv(Y)(p11.2q11.2)	15	0	0	0	0	0	0	15
Inverted Y	45,X/46,X,inv(Y)(p11.2q12)	1	0	0	2	14	0	16	17
	inv(Y)(p11.2q12)	0	0	0	0	2	0	2	2
Inverted X	inv(X)	1	0	0	0	0	0	0	1
Yqs[a]	46,X,Yqs	4	0	0	0	0	0	0	4
Add X	46,X,add(X)	0	0	0	0	0	3	3	3
Autosomal inversion	46,XY,inv(a[b])	23	0	0	0	0	0	0	23
Sex reverse	46,XX	0	0	0	0	0	38	38	38
	Mosaic	0	0	0	0	0	4	4	4
Chimerism	Chi 46,XX/46,XY	2	0	0	0	0	0	0	2
Turner syndrome	45,X	0	0	0	0	0	2	2	2
	45,X/46,XY	22	0	0	3	6	0	9	31
	45,X/46,X,r(Y)	4	0	0	1	0	0	1	5
	45,X/46,X,del(Y)/46,XY,r(Y)	0	0	0	0	1	1	2	2
	45,X/46,X,del(Y)	0	0	0	1	6	2	9	9
	45,X/46,XY/47,XYY	6	0	0	1	1	0	2	8
Jacobs syndrome	47,XYY	29	0	0	0	0	0	0	29
	45,X/47,XYY	4	0	0	0	0	0	0	4
Down syndrome	47,XY,+21	2	0	0	0	0	0	0	2
Klinefelter syndrome	47,XXY	944	0	0	0	0	0	0	944
	Mosaic	95	0	0	0	0	0	0	95
Marker	47,XY,+mar	8	0	0	0	0	0	0	8
	47,XY,+mar/46,XY	6	0	0	0	0	0	0	6
Autosomal rings	46,XY,r(A[c])	2	0	0	0	0	0	0	2
Translocations	46,X,t(A;A)	84	0	0	0	0	0	0	84
	46,X,t(X;A)	9	0	0	0	0	0	0	9
	46,X,t(Y;A)	16	0	0	0	1	0	1	17
	46,X,t(X;Y)	1	0	0	0	0	0	0	1
	Robertsonian translocations	61	0	0	0	0	0	0	61
Add Y	46,X,add(Y)	3	0	0	0	0	0	0	3
Der Y	45,X,der(Y;A)	0	0	0	0	3	2	5	5
	der(Y;Y)	1	0	0	0	0	0	0	1
Y Deletion	46,X,del(Y)	2	0	0	4	14	7	25	27

Table 3. Continued

Chromosomal status	Karyotype	Without YCMs	With YCMs						Total
			AZFa	AZFb	AZFc	AZFbc	AZFabc	All types of YCMs	
Isodicentric Y	46,X,idic(Y)(q11.2)	1	0	0	0	11	0	11	12
	45,X/46,X,idic(Y)(q11.2)	1	0	2	1	35	0	38	39
	45,X/46,XY/46,X,idic(Y)(p11.2)	0	0	0	1	0	0	1	1
	45,X/46,X,idic(Y)(p11.3)	6	0	0	0	0	0	0	6

AZF: azoospermia factor, YCM: Y chromosome microdeletion.
[a]Satellited Y chromosomes.
[b]Autosomes of 1, 4, 6, 7, 11, 12, 13, 14, and 18.
[c]Autosomes (1–22).

5. Patients lacking chromosomal aberrations or YCMs

In 78.2% (7,931/10,137; 95% CI, 77.4–79.0) of the cases studied, genetic screening revealed a normal 46,XY karyotype and no YCMs, thus in almost 3 out of 4 patients the etiology of the spermatogenesis failure remained undetermined even after extended and advanced evaluations (Table 3).

DISCUSSION

The results obtained from karyotyping and genotyping analysis of such a large cohort of patients with either NOA or SO provided the opportunity to contemplate some of the current paradoxes/paradigms in the field of androgenetics.

1. Should routine screening for YCMs be included in the clinical workup of patients with KS?

With estimated occurrence of 1 in 400–600 males across all ethnic groups and 1 in 7 in infertile cases with NOA, KS is the most common type of gonosomal aneuploidy in humans [2]. After KS, YCMs have emerged as the most frequently identified genetic cause of infertility in men [18], with global incidence of 1 in 14 (7%) in patients with NOA [19]. Thus, the role of classical YCMs in the etiology of azoospermia is indisputable and screening should be conducted in cases with NOA. However, this may not be the case for those with KS, as there are two opposing views regarding the diagnostic value of AZF screening in NOA or SO with clinically suspected KS. On one hand, some studies have demonstrated that individuals with 47,XXY karyotype may also harbor YCMs and hence conclude that they have indication for genetic screening of AZF regions [20], whereas others support that no YCMs were identified in these cases and thus exclude the need for screening [9,11]. From a diagnostic viewpoint and despite all the phenotypic diversities that have been reported in patients with KS [1], our findings coincide with the latter and support that YCMs do not occur concurrently in patients with KS, as the prevalence of YCMs among our cohort of 1,039 KS cases with both mosaic and classic forms, was nil (with statistical power of 0.95, α-error=0.03).

2. What is the indication of YCMs in cases with KS?

In line with EMQN/EAA guidelines, our results (statistical power=95%, significance level=0.03) confirm that KS prevalence is not concomitant with YCMs and hence males with KS have no indication for YCM screening. Our data moreover indicates that this is not limited only to KS, but extends to autosomal inversions/translocations, Robertsonian translocations, and Jacobs's syndrome (as described in the subgroup 4 of the present analysis), that encompass more than two-thirds of all cases with chromosomal aberrations. The underlying reason(s) for these observations is still not completely clear and a question for the future.

3. Potential factors that may introduce discrepancies in the prevalence of YCMs in men with KS

The frequency of YCMs among azoospermic males with KS has showed marked discrepancies between different studies, ranging from nil to 66.7% (Table 1). Some of the potential confounders include (but are not limited to) diagnostic protocols, sample size or selection criteria for sampling, ethnic/geographic differences [9,21-23]. It is suggested that the composition of the study population, as for example the clinical context of recruited cases

from a tertiary-referral fertility center in comparison with other settings with less-selective referrals, is the main confounder, causing a wide range of deletion rate among different research works [12,21]. The adopted diagnostic protocols, in terms of the utilized STS/gene markers, may be the next most-significant confounder. According to the literature, there has been an interest for applying additional STSs, over-and-above those recommended by EMQN/EAA guidelines, in hope to increase the resolution of screening/mapping and hence likelihood of identifying the YCMs (Table 1, Supplement Table 1). Herein, it is noteworthy to mention that YCM rate is independent of the number of STSs used [19]. Furthermore, STSs might act as a double-edged sword and have risks along with the rewards and this is mainly due to their differing clinical significance in male factor infertility assessment. Applying polymorphic/repetitive markers for STS-PCR screening, may lead either to false negative/positive PCR results (*e.g.*, sY109, sY124, sY130, sY132, sY133, sY153, sY155, and sY164) [12,18,19,24], thus potentially challenge the methodologically-derived accuracy of the reported results [8-12,20,23,25-38]. To this regard, EMQN/EAA guidelines have previously implied a likelihood of bias introduction in the studies showing a high incidence of YCMs in KS patients due to the applied STSs of choice. Since such deletions have not been confirmed with additional independent analyses the observed deletions are probably to be "methodological artifacts" [12].

4. The frequency of YCMs in Iranian population

After the report from Simoni et al (2008) of a markedly high frequency of YCMs detected in Iranian infertile patients (24.2%), some aspects should be apposed as to this notable difference in comparison with other countries worldwide [33]. Firstly, the reported data derives from a study with a limited sample size (99 participants) from a focal geographical recruitment of northwest Iranian population, thus lacking generalizability of the results for other parts of the Iranian country and its local populations [39]. Subsequent studies with larger cohorts admitted to a tertiary referral infertility clinic reported a much lower prevalence of about 5% for YCMs [13,40], results that coincide with data presented by Simoni et al [19] that provided estimates of around 7.3% of infertile Caucasian patients presenting with AZF deletions along the Y chromosome.

5. The indications for YCM screening and karyotyping in patients with NOA according to the EMQN/EAA guidelines

The next points addressed are the patient management priorities according to the EMQN/EAA guidelines in the context of applicable genetic tests during infertility workup. AZF screening is recommended as the first-tier test during infertility genetic workup for all cases with sperm count of $<5\times10^6$/mL, while karyotyping is indicated only when AZFc or Yq terminal deletions are present to rule out 46,XY/45,X mosaicism, as an extension analysis. In contrast, the same guidelines state that cases with an abnormal karyotype, except 46,XY/45,X, have no indication for AZF screening [12]. Hence based on these guidelines, it is not clear which one of the genetic tests should be requested by clinicians first.

On this basis, it is worthwhile to mention that there appear to be two different schools of thought with clinical geneticists on one hand and clinicians/urologists on the other. Although from a clinical genetics perspective, there may be a role for ordering a karyotype before YCM testing, in routine clinical practice, physicians treating patients with NOA or SO usually request both karyotype and AZF assessment at the same time, regardless of what has been recommended by the guidelines. This is done not only to improve patient satisfaction by reducing wait times before starting infertility treatments, but also because of the fact that the provided guidelines are not so crystal clear. This may be due to the paucity of literature on the interconnection of the genetic markers used in making the diagnosis. Therefore, guidelines should be either clear or adopt simultaneous testing.

6. What is the occurrence of YCMs in association with sex chromosome mosaicism?

In the literature, some studies report higher prevalence of YCMs in cases of sex chromosome mosaicism (*e.g.*, 45,X/46,XY or 47,XXY/46,XX) [41,42]. To this regard, EMQN/EAA guidelines have highlighted a cross-talk between 45,X/46,XY mosaicism and YCMs and it has been suggested that extended YCMs act as a predisposing factor in Y chromosome instability and development of 45,X cell line on the mosaic individual [43]. Our data suggests that while patients with some types of sex chromosome mosaicisms such as 45,X/46,X,del(Y); 45,X/46,X,inv(Y)(p11.2q12); or even 45,X/46,XY (odds

ratio, 8.4; 95% CI, 3.8–18.3) have indeed a higher prevalence of YCMs, this does not appear to apply in individuals carrying 47,XXY/46,XX mosaicism, as none of the 32 patients with this mosaicism (irrespective to the prevalence of each cell line) had any YCMs following screening (Table 3).

7. Is there any indication that the inv(Y) is associated with YCMs?

Whether inv(Y) is associated with YCMs, largely depends on the breakpoints. The pericentric inversion of Y, inv(Y)(p11q11), is generally known as a normal polymorphism with an overall prevalence of 0.1%–0.2%; although the rate varies depending on the population studied [44]. The real prevalence of inversion in the general healthy population is unclear, since the data resulted from epidemiological analysis of infertile cohorts with an indication for karyotyping. In our study population, the detected frequency was in accordance with previous reports from literature. Regarding the association between inv(Y) and YCMs, the prevalence in cases with inv(Y)(p11q11) was nil; however, it was not the case when the breakpoint was located at q12, inv(Y)(p11.2q12), or 45,X/46,X,inv(Y)(p11.2q12), as all were positively screened to carry AZFbc/AZFc deletions except from only one case.

8. Are chromosomal heteromorphisms clinically insignificant?

The presence of inv(Y)(p11q11) and other chromosomal heteromorphisms amongst the cohort of males with severe spermatogenesis defects, may be considered as a coincidental event or echoes some previous findings on the etiological role of chromosomal polymorphic variations in infertility [44]; however, to achieve a definite conclusion, further pieces of evidence are needed from future studies.

9. The association between sperm concentration and YCMs in oligozoospermic men

Our data revealed that only 1% of men with sperm concentration of $>1\times10^6$ sperm/mL had evidence of YCMs which is in accordance with data reported for North America and Europe [24]; These findings altogether reflect the need for reconsidering the thresholds of sperm concentration in male infertility guidelines as an indicator for YCM screening during infertility workup.

It may also be worth mentioning that herein (Table 2), two severe oligozoospermic men are represented with AZFb and AZFbc deletions, two forms of YCMs usually associated with Sertoli cell-only syndrome and maturation arrest. The observation of such deletions in cases with presence of spermatozoa in ejaculate is scarce with only scattered cases reported in the literature [45,46]. Hence, despite what is currently recommended in clinical practice [45], the chance of sperm retrieval among men with AZFb/AZFbc deletions is not nil, and such possibility should be considered in future guidelines and individually when counseling infertile couples with male counterparts carrying such YCMs.

10. The role of haplogroup distribution in Y-DNA research?

Haplotype refers either to the mitochondrial DNA (mtDNA) or to the non-recombining regions of the Y chromosome with sequence polymorphism variations, excluding the pseudoautosomal Y regions. The mtDNA is maternally-derived (contributed from the oocyte), while the latter is inherited paternally. This unusual pattern of inheritance has been utilized as a DNA signature to trace direct maternal or paternal ancestral lineages, respectively [47]. Haplogroup is therefore defined as a set of nearly identical haplotypes sharing a common ancestor.

One of the major concerns in the field of "Y chromosome research" is population stratification vis-à-vis homogeneity in haplogroup distribution, and its role as a potential confounding element. This is because, from a population genetics viewpoint, it has already been shown that even seemingly homogeneous cohorts clustered using mathematical algorithm (principal-component analysis) still suffer from differences in Y-DNA haplotypes which stem from the population history [48]. Moreover, it was hypothesized that Y-DNA haplogroups may play a predisposing/protecting role in the prevalence of YCMs, although, the results are conflicting rather than conclusive. While a double-blind study in 138 consecutive men of European descent failed to find any evidence to support the concept [21], another study revealed that in comparison to other haplogroups, those with haplogroup E are more prone to AZFc (b2/b4) microdeletions [22].

11. Strengths and limitations of the current study

As the current study was conducted in a predominantly Iranian population, one may argue about the uncertainty surrounding the interpretation of the results in terms of their external validity and generalizability to other geographic/ethnic groups (Y chromosome haplotypes). It is noteworthy to mention that the current study takes advantage of two key attributes that could, at least in part, exclude this possibility. The first is that there is an inherently high haplogroup diversity in the Iranian population (0.952) owing to the different migratory events during history [49]. The second is the moderate heterogeneity of the studied population concerning its ethnic composition, as 20 out of 578 cases with YCMs (3.5%) were not of Iranian origin: 55% (11/20) were Afghan, 20% (4/20) were Iraqi, 10% (2/20) were from Azerbaijan, and 15% (3/20) were from Germany, Oman, and United Arab Emirates, respectively. Moreover, as other single-center trials, the study benefits from a relatively high-degree of uniformity in the applied procedures over the study period, both in terms of scientific personnel experience and standardized clinical practice but also in terms of equipment and scientific instrumentation.

In the current study, we assess the rate of concomitant occurrence of two apparently irrelevant genetic alterations, in the largest cohort of infertile men presented to this day. Although the study was conducted in such a way that the results could be extrapolated to greater proportion of ethnicities, the extension of the outcomes beyond the target population is still a concern. Confirmatory studies in other geographic/ethnic groups are still needed to reach a consensus regarding the outcomes. Moreover, considering the fact that the type/prevalence of chromosomal aberrations detected in lymphocyte cultures does not necessarily reflect the karyotypic situation in target tissues, applying molecular cytogenetic approaches for comprehensive chromosomal screening of the testis would be of great value particularly for those with normal karyotype.

CONCLUSIONS

The outcomes of this study can be summarized as follows:

1. Patients with AZFb or AZFbc microdeletions may undergo spermatogenesis with reduced spermatozoa populations in the ejaculate.
2. Only 1% of the screened population with a sperm concentration of $>1\times10^6$ sperm/mL had AZF microdeletions, therefore testing referral for such populations should be reviewed in future guidelines.
3. Mosaic and classic forms of KS do not indicate YCMs screening due to the null prevalence detected in KS patients.
4. In contrast to previous recommendations, our findings demonstrate that along with cases of mosaic monosomy X karyotype, patients with the following chromosomal abnormalities inv(Y)(p11.2q12); idic(Y)(q11.2); 46,XY,r(Y); idic(Y)(p11.2) and der(Y;Autosome) (76/169; 44.9%; 95% CI, 37.7–52.5) should accordingly be referred for AZF deletion screening.
5. Despite previous reported divergences in the prevalence of YCMs in the Iranian population, our large-scale findings demonstrate that the YCMs prevalence lies at approximately 5% of the general Iranian population.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: HK, MS. Data curation: HK. Formal analysis: HK, PV, AR. Investigation: HK. Methodology: HK, MS. Project administration: MS, MSG. Resources: HK. Supervision: MS, MSG. Validation: MS, PV, AR, AA, MSG, GMC. Visualization:

HK. Writing – original draft: HK. Writing – review & editing: All authors.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article (and its supplementary files).

Supplementary Material

Supplementary material can be found *via* https://doi.org/10.5534/wjmh.220089.

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