ORIGINAL ARTICLE

AZFa, AZFb, AZFc and gr/gr Y-chromosome microdeletions in azoospermic and severe oligozoospermic patients, analyzed from a neural network perspective

Microdeleciones de las regiones AZFa, AZFb, AZFc y gr/gr del cromosoma Y en pacientes con azoospermia y oligozoospermia severa, análisis desde una perspectiva de red neuronal

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Abstract

Aim: *Analysis of male infertility by molecular methods has increased since recognition of genetic risk factors. The AZFa, AZFb, AZFc, and gr/gr regions on the Y-chromosome can cause male infertility. The aim of this study was to determine the prevalence of Y-chromosome microdeletions in these regions in infertile Mexican patients.* **Material and methods:** *We recruited 57 infertile patients with abnormal sperm count (26 azoospermic and 31 oligozoospermic) and 55 individuals with normal sperm count. Analysis of the regions of interest was performed by PCR.* **Results:** *15.8% of infertile patients presented Y-chromosome microdeletions, whereas no deletions were found in the control group. Deletions were observed in all the analyzed regions except in AZFa. Additionally, the neural network model revealed a mild genotype-phenotype correlation between deletion of the sY1191, sY1291 and sY254 markers with oligozoospermia, azoospermia and cryptozoospermia, respectively.* **Conclusions:** *Our data show that AZFb, AZFc, and gr/gr microdeletions are significantly associated with infertility in Mexican population. In addition, the neural network model revealed a discrete genotype-phenotype correlation between specific deletions and a particular abnormality. Our results reinforce the importance of the analysis of AZF regions as part of the clinical approach of infertile men.*

Keywords: *Male infertility. Y-Chromosome microdeletions. AZF region. Azoospermia. Oligozoospermia.*

Resumen

Objetivo: *La utilización de técnicas moleculares para estudiar la infertilidad masculina se ha incrementado desde el reconocimiento de factores genéticos. Las regiones AZFa, AZFb, AZFc, y gr/gr del cromosoma Y son causa de infertilidad masculina. El objetvo de este estudio fue determinar la prevalencia de microdeleciones en estas regiones en pacientes infértiles*

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Mexicanos. **Material y métodos:** *Reclutamos 57 pacientes infértiles con cuentas espermáticas anormales (26 con azoospermia y 31 con oligozoospermia) y 55 individuos con cuentas espermáticas normales. El análisis de las regiones se realizó mediante PCR.* **Resultados:** *15.8% de los pacientes infértiles presentó microdeleciones, no se encontraron microdeleciones en el grupo control. Las microdeleciones fueron observadas en todas las regiones excepto en AZFa. Adicionalmente, el modelo de red neuronal reveló una leve correlación genotipo-fenotipo entre microdeleciones de los marcadores sY1191, Sy1291 y sY254 con oligozoospermia, azoospermia y criptozoospermia, respectivamente.* **Conclusiones:** *Nuestros datos muestran que las microdeleciones en AZFb, AZFc, y gr/gr se asocian significativamente con infertilidad en la población Mexicana. Además, el modelo de red neuronal reveló una discreta correlación genotipo-genotipo entre microdeleciones específicas con una anormalidad en particular. Nuestros resultados refuerzan la importancia del análisis de las regiones AZF en el abordaje de la infertilidad masculina.*

Palabras clave: *Infertilidad masculina. Microdeleciones del Cromosoma Y. Región AZF. Azoospermia. Oligozoospermia.*

Introduction

Approximately 7% of men present fertility problems during their lives, resulting in a public health issue[1,2]. Male infertility is a complex pathology defined by the failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse, according to international consensus[3]. Male contribution to infertility represents 45%–50% in childless couples[4,5]. Diagnosis is made based on abnormal semen analysis according to the WHO criteria. Based on sperm count, patients can be classified as azoospermic, defined as the absence of sperm in at least two different ejaculate samples (including the centrifuged sediment), or oligozoospermic when sperm account is <15 million/mL. In addition, severe oligozoospermia is considered with sperm concentration <5 million/mL and criptozoospermia when sperm is only visualized after centrifugation[6]. Azoospermia is classified into two groups: 1) obstructive, caused by obstructive factors along the seminal pathways, and 2) secretory or non-obstructive azoospermia (NOA), which is commonly related to gonadal dysfunction. NOA is explained by genetic factors in 21%–29% of cases, whereas in 12%–41% of cases, the cause is idiopathic and probably related to unknown genetic factors[7-9]. The main genetic cause involved in male infertility are abnormalities of the Y chromosome[10], such as numerical and structural alterations. In addition, epigenetic modifications, such as decreased DNA methylation of the IGF2-H19 region, autosomal gene mutations, i.e. in the gene CFTR and in the X chromosome, the CAG polymorphism in the androgen receptor gene, have also been involved[11-13]. Of interest is the azoospermia factor region (AZF region) localized on Yq, which contains genes involved in the control of male meiosis[14]. Six AZF regions are known, AZFa, AZFb, AZFc, and their combinations[15]. Non allelic homologous recombination between repetitive sequences can lead to chromosome deletions or duplications, this mechanism is believed to account for the random appearance of *de novo* AZF microdeletions[16]. AZF microdeletions have been proved to be in a clear cause-effect relationship with spermatogenic impairment[14,17,18]. Microdeletions are detected in 10%–15% of patients with NOA and 5%–10% of patients with severe oligozoospermia[19,20]. The most frequent deletion occurs in the AZFc region (~80%), followed by AZFb (1%–5%), AZFbc (1%–3%) and AZFa (0.5%–4%)[21]. Patients harboring deletion of AZFa can present hypospermatogenesis and inhibition of the production and maturation of germ cells[22,23]. The phenotype observed in patients with AZFb deletion is a pre-meiotic arrest with normal spermatogonia and primary spermatocytes. AZFb overlaps with the AZFc region[24]. The AZFc region contains critical transcripts for normal spermatogenesis. Spermatid or sperm maturation anomalies have been found in patients with AZFc microdeletions[17].

While the analysis of AZF deletions have been introduced as a routine genetic test for infertile patients, the role of partial AZFc deletions, i.e., gr/gr deletion, b1/b3, and b2/b3, has been the focus of long-lasting debate. The risk associated with this genetic anomaly varies between populations, reaching the highest OR in Italians (OR 7.9, 95% CI 1.8–33.8)[25]. Current evidence suggest that the gr/gr deletion is a significant risk factor for impaired sperm production.

We designed the present study to analyze the frequency of microdeletions in the AZF region in a group of Mexican mestizo infertile men.

Materials and Methods

Patients

This study was performed at the Genetics and Genomics Department of the National Institute of Perinatology

in Mexico during a four years period, all procedures were approved by the institutional ethics committee. Patients with idiopathic infertility were referred from the Andrology Department. Participants were invited to the study and signed an informed consent form. A total of 57 patients were included, 26 had azoospermia and 31 had severe oligozoospermia (12 criptozoospermic and 19 oligozoospermic), all of them underwent to a GTG karyotype. The control group was composed by 55 males with normal spermatobioscopy.

Molecular analysis

Genomic DNA was isolated from peripheral blood with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer´s instructions. A NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used to determine DNA purity and concentration.

Two multiplex PCR reactions were performed to analyze the AZFa, AZFb, and AZFc regions and singleplex PCR reaction to analyze the gr/gr region, as described elsewhere[18]. The multiplex PCR reaction employed the primers ZFX/Y, sY86, sY127, sY254 and the singleplex used the primers sY14 (SRY), sY84, sY134, and sY255. We confirmed the deletions using a single PCR reaction. For amplification of the gr/gr region, we used the primers sY1291 and sY1191. The heterochromatic Yq region was analyzed with the sY160 marker. The list of primer sequences, and expected product sizes has been previously published[18].

Each multiplex or singleplex PCR reaction contained 0.7–1 μg of DNA, 200 nM of each primer and 1.5 units of HotStarTaq Master Mix Kit. Cycling conditions were as previously described[20]. PCR products were size separated by electrophoresis in a 2.5% agarose gel (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Descriptive statistics are presented for all variables. Kruskall Wallis test was performed to evaluate quantitative variables. Pearson`s χ^2 test was used to compare proportions between study groups.

A multilayer perceptron neural network model was executed to determine whether the presence of a determined deletion could predict azoospermia, criptozoospermia, or oligozoospermia. A classification and regression tree (CRT) was later used to corroborate neural network predictions and as a predictive model for azoospermia, cryptozoospermia, or oligozoospermia, according to the presence of deletions in the different regions analyzed. Lastly, another CRT model was executed to determine whether the number of deletions in the evaluated regions could be related to any of the three diagnoses. Statistical analyses were performed using IBM SPSS Statistics (Armonk, NY, USA), version 22. The significance level was $\alpha = 0.05$.

Table 1. Percentage of patients and controls with and without microdeletions

Group	Without deletion n (%)	With deletion n (%)	Total	P*
Patients	48 (84.2%)	9 (15.8%)	57	0.002
Controls	55 (100%)	0	55	
Total	103 (92.0%)	9 (8.0%)	112	

*Statistically significant difference between patients and controls by Pearson χ^2.

Results

We analyzed 55 individuals with normal spermatobioscopy as controls and 57 diagnosed with infertility. All participants had a normal karyotype. Within the infertility group, 26 were azoospermic (45.6%) and 31 were severely oligozoospermic, from these, 12 (21.1%) were criptozoospermic and 19 (33.3%) oligozoospermic. The mean age of participants was 32.7 (SD 6.7) years and mean semen sample volume was 2.56 (SD 1.86) mL. Kruskall Wallis tests showed no significant difference in participants' age and semen sample volume among the different diagnoses (azoospermia, cryptozoospermia, and oligozoospermia).

None of the controls presented microdeletions within the STSs analyzed. We found a microdeletion in 9 (15.8%) individuals from the infertility group. When we compared the proportion of individuals with microdeletions through a Pearson X^2 test we observed a statistically significant difference (p=0.002) (Table 1).

Table 2 shows the number of patients with microdeletions according to the clinical diagnoses within the infertility group. We did not find statistically significant differences when comparing individuals with azoospermia versus severe oligozoospermia (Pearson X^2 test, p=0.615).

Among azoospermic patients, one had a complete deletion of the AZFb region (absence of sY127 and sY134 markers; Figure 1A and B, patient AZP3). Another had a complete deletion of the AZFc region (absence of sY254 and sY255 markers, AZP4) (Fig. 1A and B). Two

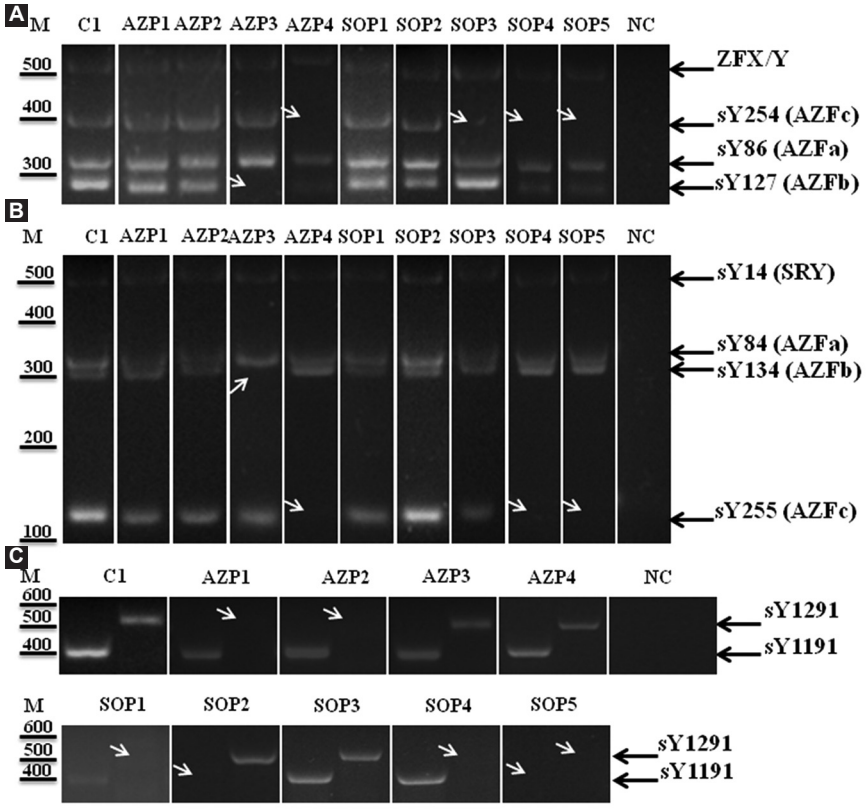

Figure 1. *AZFa, AZFb, AZFc, and gr/gr microdeletions in azoospermic or severe oligozoospermic Mexican patients.* **A:** *representative image of multiplex PCR reaction A to analyze the presence/absence of STS sY86 (AZFa), sY127 (AZFb) and sY254 (AZFc), and the positive control ZFX/Y.* **B:** *representative image of multiplex PCR reaction B to analyze the presence/absence of STS sY84 (AZFa), sY134 (AZFb) and sY255 (AZFc), and the positive control sY14 (SRY).* **C:** *representative image of singleplex PCR reactions to analyze the presence/absence of STS sY1291 and sY1291. White arrows show the presence of microdeletion. AZP: azoospermic patients, C: controls, NC: negative controls, SOP: severe oligozoospermic patients.*

Table 2. Proportion of azoospermic or severe oligozoospermic patients with microdeletions

Diagnosis	Without deletion	With deletion	Total
Azoospermic	22 (84.6 %)	4 (15.4%)	26
Severe oligozoospermic			
Criptozoospermic	9 (75%)	3 (25%)	12
Oligozoospermic	17 (89.5%)	2 (10.5%)	19
Total	48 (84.2%)	9 (15.8%)	57

No statistically significant differences were observed between azoospermic and severe oligozoospermic patients by Pearson χ^2 (p = 0.615).

of them had the gr/gr microdeletion (absence of sY1291 and presence of sY1191 markers, patients AZP1 and AZP2) (Fig. 1C). Within the severe oligozoospermic group, two patients had a complete deletion of the AZFc region (Fig. 1A and B) (patients SOP4 and SOP5). Patient SOP4 presented absence of the sY1291 gr/gr marker (Fig. 1C), while patient SOP5 had absence of both gr/gr markers (Fig. 1C). One patient had a partial microdeletion of AZFc (absence of sY254 marker, SOP3) (Fig. 1A). Another had a complete gr/gr microdeletion (absence of sY1291 marker, SOP1) (Fig. 1C). Another patient presented a partial gr/gr microdeletion (absence of sY1191 marker, SOP2) (Fig. 1C). Table 3 summarize microdeletions findings.

A multilayer perceptron neural network model using the sigmoid activation function was performed to determine if the presence of deletions in the sY127, sY134, sY254, sY255, sY1291, or sY1191 regions were associated with azoospermia, cryptozoospermia, or oligozoospermia. The data were divided randomly into a training group (70%) and a holdout group (30%) with n1/n2 cross-validation to evaluate the accuracy of the network model. The model was re-run several times with different random starting seeds to ascertain that the results were consistent. The area under the curve for azoospermia, cryptozoospermia, and oligozoospermia in the training set were 0.640, 0.643, and

Table 3. Summary of Y-chromosome microdeletions found in infertile patients

Diagnosis	AZFb		AZFc		gr/gr	
	SY127	SY134	SY254	SY255	SY1291	SY1191
Azoospermic AZP1					X	
Azoospermic AZP2					X	
Azoospermic AZP3	X	X				
Azoospermic AZP4			X	X		
Severe oligozoospermic SOP1 (Criptozoospermic)			X			
Severe oligozoospermic SOP2 (Criptozoospermic)					X	
Severe oligozoospermic SOP3						X
Severe oligozoospermic SOP4 (Criptozoospermic)			X	X	X	
Severe oligozoospermic SOP5			X	X	X	X

0.635, respectively. The model accurately predicted 54.3% of training cases. The regions with the highest importance to the model were sY254 (100%), sY1191 (99%), sY1291 (66.8%), and sY255 (65.2%). The predicted values for deletions in the regions with highest importance to the model according to diagnosis are presented in Figure 2. The model predicted that deletions in sY1191 were more likely related to oligozoospermia, while deletions in sY1291 were more related to azoospermia and deletions in sY254 were more related to cryptozoospermia. A follow-up CRT decision tree indicated that deletions in the sY1191 region were mainly associated with oligozoospermia (n = 2, 100%), whereas the absence of deletion in sY1191 and the presence of deletion in sY254 were mostly related to cryptozoospermia (n = 2, 66.7%) and to azoospermia (n = 1, 33.3%). On the other hand, neither deletion in sY1191 nor deletion in sY254, accompanied by a deletion in sY1291 was mainly related to azoospermia (n = 2, 66.7%) and to cryptozoospermia (n = 1, 33.3%) (data not shown). The most important predictive variables in the model were sY1191, sY254, and sY1291. It is noteworthy that none of the patients with azoospermia or cryptozoospermia had deletions in sY1191.

Finally, a CRT model was executed to determine if not only the presence of deletion for each region but the total number of deletions could predict any of the three diagnoses. The tree indicated that the most important independent variable predicting diagnosis was the total number of deletions (normalized importance of 100%), followed by sY1191. The model showed that patients with deletions in the sY1191 region were more likely to have oligozoospermia, but patients with no deletion in the sY1191 and one or more deletions in any of the other regions were more likely to belong to the azoospermia or cryptozoospermia groups (57.1% and 42.9%, respectively). The decision tree is shown in figure 3.

Discussion

We investigated Y-chromosome microdeletions in patients diagnosed with infertility. We screened microdeletions using STS markers for the AZFa, AZFb, AZFc, and gr/gr regions. Regardless of deletion type, the overall deletion frequency in azoo-/severe oligozoospermic men was higher than in normozoospermic (15.8% vs. 0). The statistical analysis showed that microdeletions are significantly associated with infertility in our population ($p = 0.002$). This result suggests that such deletions could be a risk factor for impaired spermatogenesis in the Mexican population. There are few studies in Mexican populations analyzing the prevalence of Yq deletions in infertile men. Piña et al, studied the presence of Yq microdeletions in Mexican men from couples with recurrent pregnancy loss, they did not find microdeletions[26]. Martinez-Garza S.G, et al found that 12.2% of infertile males had microdeletions of AZFb or AZFc, however they did not analyze gr/gr microdeletions[27]. Our results, including gr/gr deletions, showed a higher percentage (15.8%), compared to their results. It is noteworthy that they used other STS markers, they found two patients with deletion of sY121 and/or sY128 in AZFb. However, the

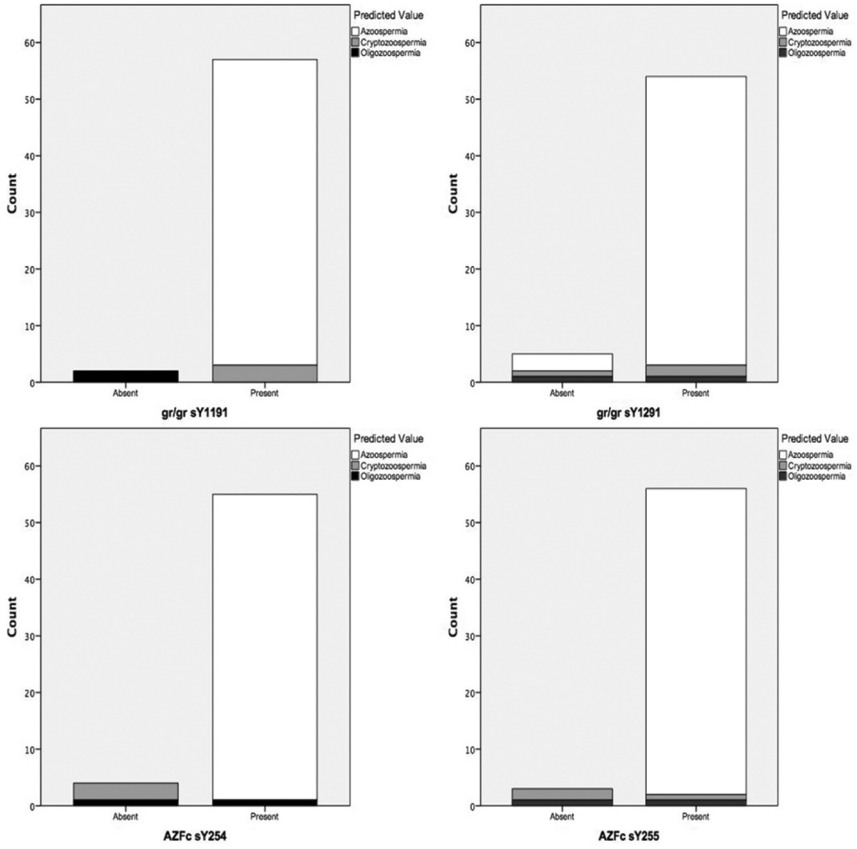

Figure 2. *Stacked bar charts of the predicted values for azoospermia, oligozoospermia, and cryptozoospermia according to microdeletions obtained from a neural network model. The top left bar chart shows that the model predicted that patients with the sY1191 deletion were more likely to be oligozoospermic. The top right bar graph shows that the model predicted that patients with sY1291 deletions were more likely to be azoospermic. The bottom charts show that the model predicted that patients with sY255, and especially those with sY254, were more likely to be cryptozoospermic (criptozoospermic and oligozoospermic).*

aforementioned markers are not included in the EAA/EMQN Guidelines 2013[20]. These STS markers could be important in the analysis of Mexican populations and could be analyzed in future studies.

The impact of gr/gr deletions on male fertility is unclear. Gianchini et al, found that the frequency of gr/gr deletions was significantly higher in the infertile group compared to controls, suggesting a possible deleterious effect on spermatogenic efficiency. They reported an OR of 10.1, indicating that gr/gr deletions could be considered a risk factor for oligozoospermia[28]. However, other studies have not detected strong association. Hucklenbroich et al, did not find significant differences of gr/gr deletions in German population when compared men with NOA versus normospermic men[29]. A lack of association was also observed by Machev et al in Italian population[30]. They observed that 5.3% of infertile men (7/150) had gr/gr deletions compared to 0.5% of fertile individuals (1/189). Although these data suggested a significant association, from these seven patients, four were defined as idiopathic, one had a history of cryptorchidism and two had varicocele[31].

In our study, from the infertile men who only presented a complete deletion of the gr/gr region, 2/26 were azoospermic (7.7%) and 1/31 (3.2%) had severe oligozoospermia (criptozoospermia). Whereas, none of the controls presented the deletion. There were significant differences in various parameters associated with semen quality in men with gr/gr subdeletions, mainly in the sperm concentration, which further reinforces the point that these mutations may be associated with spermatogenesis impairment. This observation has been described previously[28]. To further define the genotype-phenotype correlation, we found the role of partial gr/gr subdeletions to be more complicated than previously thought and probably is related to the study population[32].

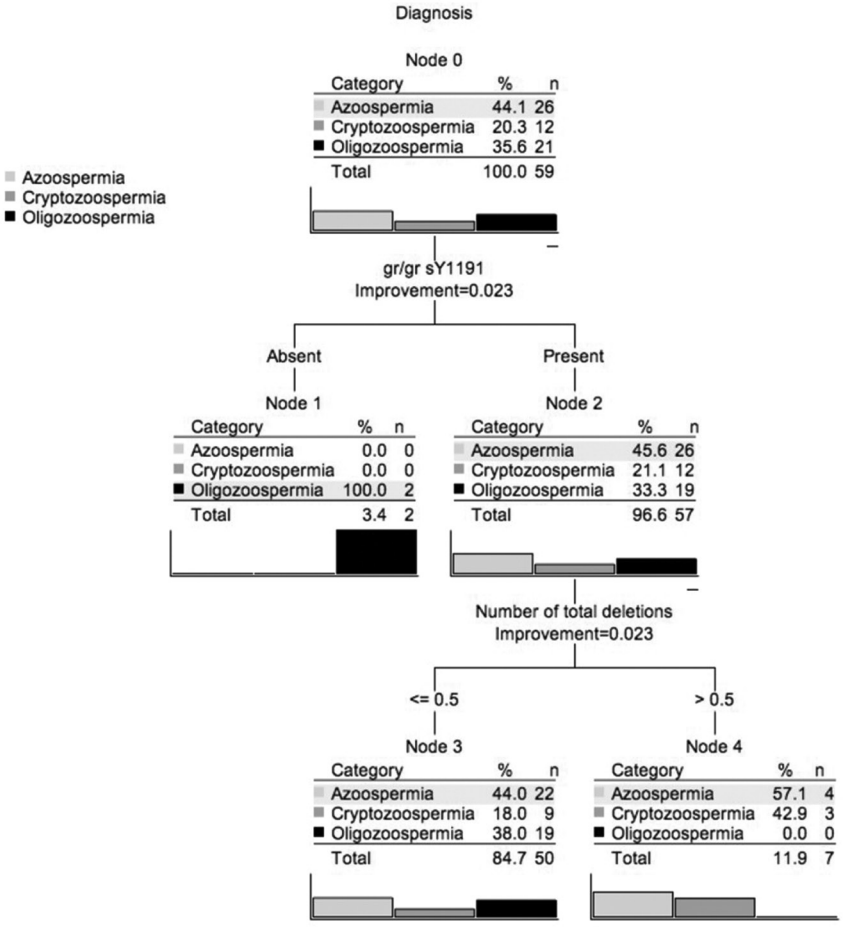

Figure 3. *Classification and regression tree. The CRT indicates that the tree split first according to sY1191 deletions and that 100% of patients with this type of deletion had oligozoospermia. However, in those patients that did not have sY1191 deletions, the most important variable defining the infertility categories was the number of deletions per participant. The final nodes in the tree indicate that patients with one or more deletions were more likely to have azoospermia or cryptozoospermia.*

The Pearson χ^2 test did not find differences in the STSs deleted between azoospermic and severely oligozoospermic patients. However, the neural network model showed that some STS deletions could be related to azoospermia, criptozoospermia, or oligozoospermia. The model showed that the sY1191 deletion could predict oligozoospermia, while the sY1291 deletion may predict azoospermia and the sY254 deletion could be related to criptozoospermia. These results were corroborated by a classification and regression tree (CRT). We also performed a CRT to determine if the number of deletions per patient could predict any of the diagnoses. Again, the CRT model showed that patients with deletions in the sY1191 region were more likely to have oligozoospermia, and patients without sY1191 deletion and one or more deletions in any of the other regions were more likely to be azoospermic or cryptozoospermic.

Although this study reports novel findings concerning the potential correlation between certain microdeletions with azoospermia, cryptozoospermia, and oligozoospermia, it has some limitations. Certainly, the sample size and the small number of microdeletions found may have influenced our results. A study with a larger sample size is necessary to confirm our results.

Future studies in larger groups should focus on the combined definition of the type and copy number of the AZFc genes deleted in men with partial deletions and the haplogroup of the Y chromosome. A detailed analysis of these and other yet unidentified genetic factors is necessary prior to offer assisted reproductive techniques.

Conclusions

Our findings corroborate the importance of microdeletions of the AZF region in infertile patients.

Despite the controversy of the gr/gr deletion as a risk factor for male infertility, we did not observe this deletion in fertile controls. More studies with lager samples in ours and other populations are needed to define the gr/gr deletion as a factor compromising fertility. Nevertheless, the study of AZF microdeletions has to be included in the clinical approach of infertile males.

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Conflict of interest

The authors report no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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