From whole exome analysis in idiopathic azoospermia to the identification of a high risk subgroup for occult Fanconi Anemia

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ABSTRACT

Purpose: In about 10% of patients affected by Fanconi Anemia (FA) the diagnosis is delayed until adulthood and the presenting symptom in these "occult" FA is often a solid cancer and cancer-treatment related toxicity. Highly predictive clinical parameter(s) for diagnosing such an adult onset cases are missing.

Methods: i) Whole-Exome Sequencing (WES) ii) Sanger sequencing of the *FANCA* gene; iii) DEB-induced chromosome breakage test.

Results: WES identified a pathogenic homozygous *FANCA* variant in a patient affected by Sertoli Cell Only Syndrome (SCOS) and in his azoospermic brother. Although they had no overt anemia, chromosomal breakage test revealed a reverse somatic mosaicism in the former and a typical FA picture in the latter. In 27 selected SCOS cases one additional patient showing compound heterozygous pathogenic *FANCA* variants was identified with positive chromosomal breakage test.

Conclusion: We report an extraordinary high frequency of FA in a specific subgroup of azoospermic patients (7.1%). The screening for *FANCA* mutations in such patients has the potential to identify undiagnosed FA before the appearance of other severe clinical manifestations of the disease. The definition of this high-risk group for "occult" FA, based on specific testis phenotype with mild/borderline hematological alterations, is of unforeseen clinical relevance.

INTRODUCTION

The severest form of male factor infertility is non-obstructive azoospermia (NOA), which occurs in approximately 1% of all men in reproductive age and in the majority of patients the etiology remains unknown.[1] Since spermatogenesis is regulated by the concerted action of >2000 genes, a large proportion of idiopathic NOA might be attributable to a not yet identified genetic defect.[2] Within the last years, Whole Exome Sequencing (WES) has proved to be a powerful tool for the discovery of causative variants in familial cases of NOA.[3–10] On the other hand, this approach has the potential to incidentally reveal undiagnosed diseases. Genes involved in stem cell proliferation and DNA repair are important not only for spermatogenesis but also for other physiological processes. For instance, the association between FA, caused by mutations in genes involved in DNA interstrand cross-link repair, and infertility is well known in humans; however, the exact testicular phenotype in FA patients has not been explored. FA arises in the large majority of cases with bone marrow failure during childhood however, in 10% of cases the diagnosis is delayed until adulthood.[11] The late diagnosis frequently occurs in those patients who present a slow progressive depletion of bone marrow hence the sign of onset may be the appearance of a cancer instead of an overt anemia. Diagnosing occult FA has a relevance also concerning cancer treatment due to acute toxicity to chemotherapy. We performed WES in an idiopathic NOA (iNOA) patient with consanguineous parents, who displayed Sertoli Cell-Only Syndrome (SCOS). Our investigation revealed a pathogenic variant in the *FANCA* gene providing an incidental diagnosis of FA during a genetic investigation for NOA in an adult man. Based on this finding we revisited all our patients with similar testis histology and selected among them those with mild alterations or borderline values of platelets and Mean Corpuscular Volume (MCV) for further screening of *FANCA* gene mutations. This approach has led to the identification of a high-risk group for adult onset FA.

MATERIALS AND METHODS

Study populations

Study population I: A 43-year-old man (04-170) with iNOA and with consanguineous parents (first-cousin marriage) was selected for WES. All known causes of azoospermia were excluded. Testis histology revealed SCOS type I (no germ cells were present in the tubules). See Table 1.

Study population II: 27 iNOA patients with testis histology of SCOS type I and SCOS type II (SCOS with germ cells in a minority of tubules), with platelets <200.000/L and MCV>85fL were selected for *FANCA* mutation screening (Table S1).

Whole Exome Sequencing (WES) in patient 04-170

Library preparation, Bioinformatic analysis and variants filtering are described in Supplementary Material.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Large deletions were analyzed using a Multiplex Ligation-Dependent Probe Amplification (MLPA) kit developed by MRC-Holland BV as we described before.[12]

FANCA Sanger sequencing analysis

Two-steps Sanger Sequencing (described by Castella et al. 2011)[12] in 27 selected iNOA patients: first, the three most commonly mutated exons (exons 13, 36, 38) in the Spanish population; second, the remaining *FANCA* exons.

Diepoxibutane (DEB)-induced chromosomal breakage test: mutation carrier NOA patients and and their brother's peripheral blood lymphocytes, as described elsewhere[13] (see also Supplementary Material).

Gene Expression Evaluation

Quantitative RT-PCR (qRT-PCR) analysis was performed to evaluate *FANCA* expression in adult testes biopsies, collected in our clinic and characterized with molecular markers for the definition of distinct germ cell types (details in Supplementary Material and Table S2).

RESULTS

Exome analysis of a NOA patient affected by SCOS

Patient 04-170 carried a total of 20477 exonic variants. Following standard filtering (described in supplementary material) and the exclusion of synonymous variants with a MAF≥0.05, we filtered for all homo/hemizygous variants that presented an in-house Index of Pathogenicity (IP) ≥0.7. Of the eight resulting variants, only one mapped to a gene with proved implication in spermatogenesis (*FANCA)* and presented the highest

pathogenic score (IP=0.9,) (Figure 1, Figure S1 and Table S3). The c.2639G>A variant maps to exon 28 of the *FANCA* gene (NM_000135.2) and its pathogenicity was proven by functional studies[12,14] . It was already shown to cause FA in a Spanish patient,[12] as well as reported in the FA Mutation Database at Rockefeller University both in homozygosis and heterozygosis in eight FA patients (Table S4). DEB-induced chromosomal breakage test, one of the gold-standard tests used in the routine practice for the diagnosis of FA, gave a positive result in a fraction of cells, indicating FA somatic mosaicism (Figure 1B). The presence of 2-3% of wild-type alleles in the NGS data (data not shown) suggests a possible mechanism of genetic revertion by back mutation. The confirmation of this hypothesis would request additional experiments using digital PCR, cloning and single clone sequencing or amplicon analysis by deep NGS[15].

Following the discovery of the variant, the patient, during genetic counseling, informed the doctor that also his brother suffered by azoospermia. The screening of *FANCA* in his brother revealed the same genotype. The man could not undergo physical examination in our clinic due to geographic distances, but according to the family doctor he has no evident skeletal abnormalities or other FA-related symptoms. DEB-induced chromosomal breakage test showed that the majority of cells resulted positive, revealing a typical complete FA picture (Figure 1B). It is interesting to note that although this man presented a mild decrease of platelets in 2011 and, as for 2013 he also displayed a mild decrease of red blood cells and leucocytes, this condition was not further explored by the family doctor. The hemogram from 2016, requested after our diagnosis, shows a pronounced decrease of all three cell types (Table 1).

FANCA mutation screening in selected iNOA patients with SCOS

From our large cohort of 1300 infertile men, 136 were classified as iNOA with testis histology of SCOS type I or type II. For 120/136 patients hematological parameters were available and we selected those (n=27) with platelets <200.000/L and Mean Corpuscular Volume >85fL (Figure S2, Table S1). Genomic DNA from peripheral blood of this specific group of 27 iNOA patients was screened for mutation firstly in exons 13, 36, 38 i.e. those exons which contain the most commonly observed mutations in Spanish FA patients and for large *FANCA* deletions through MLPA. We identified a heterozygous variant in exon 38 in patient 14-339 [NM_000135.2:c.3788_3790delTCT, p.Phe1263del)]. This analysis was followed by Sanger sequencing of the entire *FANCA* gene which revealed a second heterozygous variant in exon 39

[NM_000135.2:c.3913C>T ,p.Leu1305Phe] in patient 14-339. Both mutations were previously reported in many FA patients and are clearly pathogenic (Figure S2, Table S4). Similar to patient 04-170, DEB-induced chromosomal breakage test gave a positive result in a fraction of cells confirming mosaic FA (Figure S3). The brother of this patient showed normozoospermia and his hormone levels, testis volume and blood count were within the normal range (Table 1). Sanger Sequencing revealed only one of the two variants in heterozygosis [NM_000135.2:c.3913C>T, p.Leu1305Phe].

FANCA expression in the testis*:* qRT-PCR analysis was performed in a collection of testis biopsy samples (not belonging to the patients' testis biopsies due to the lack of material recollection) previously characterized by a molecular genetic approach (see Supplementary Material). *FANCA* was undetectable in SCOS, but showed the highest expression in biopsies with a prevalent spermatogonial arrest (SGA) and obstructive azoospermia (i.e. presence of all spermatogenesis stages). This expression pattern is compatible with a prevalent expression in spermatogonial and early spermatocytic stage (Figure S4). .

DISCUSSION

The large majority of FA patients are diagnosed in childhood due to the presence of significant birth defects. About 10% of patients the diagnosis is delayed until adulthood,[11] especially when they appear normal or have subtle findings that may be overlooked. The late diagnosis frequently occurs in those patients who present a slow progressive depletion of bone marrow hence the sign of onset may be the appearance of a cancer instead of an overt anemia. According to the FA Guidelines,[16] "male infertility" is one of those adult-onset signs/symptoms which should raise suspicion of FA, hence should prompt clinicians to perform peripheral blood testing. However, while FA is a rare disease (prevalence of 1/160.000), infertility is a common pathological condition, present in 7% of the general male population. Moreover, it is an extremely heterogeneous condition ranging from the complete absence of spermatozoa in the ejaculate (azoospermia) to decreased sperm number or motility/morphology.[1] Even the NOA phenotype can be further sub-classified according to the testis histology e.g. complete absence of germ cells (SCOS), spermatogenic arrest (at different stages) or hypospermatogenesis. Therefore, referring to "infertility" as a possible FA sign is extremely broad and requires a more precise definition in the clinical practice.

Our first patient, diagnosed through WES, presented borderline values of blood counts and besides infertility showed another potential FA sign, mild dysmorphic facies. His brother with the same reproductive problem carried the same genotype and as expected, the chromosomal breakage test confirmed FA in both brothers. A picture of mosaic FA in patient 04-170 and a complete FA in his brother who did not show dysmorphic features but displayed a progressive impairment of hematopoiesis reaching to pathologically low values at the control in 2016 (performed after our diagnosis).
The third patient presented low stature and some *cafe au lait* spots in his hands and back; however, FA was not suspected until he came to our clinic and was diagnosed with azoospermia. Similar to patient 04-170, DEB test showed a picture of mosaic FA also in this man. The Patient 14-339's brother carried only one of the variants in heterozygosis and his testicular function was normal. A common feature of all three affected men were their testis phenotype i.e. SCOS.
In this study, we report an unexpectedly high frequency of FA (2/28=7.1% or 3/29 = 10.3% if we consider also the brother) in a specific subgroup of iNOA subjects with testicular phenotype of SCOS type I or type II and with slightly altered/borderline hematological parameters. Our study indicates that andrological evaluation, especially in SCOS patients, should not only include hormone measurement but also blood count, since it may lead to the diagnosis of unsuspected FA in adulthood. It further supports that FA is not an exclusive childhood illness, and diagnosis and treatment are no longer exclusively performed by pediatricians. We propose that the evaluation of hematological parameters, in particular platelet count and MCV in NOA patients should be included in the routine andrological workup. *Vice versa*, we provide a more precise andrological criteria to all clinicians, about the type of "infertility" subgroup in which the likelihood of FA is relatively high. The combined assessment of testis histology and hematological parameters allows the diagnosis of "occult" FA before the appearance of severe pathologies related to this disease (e.g. hematological malignancies and specific solid cancers). It has been reported that in more than 20% of patients with FA who developed solid tumors, the diagnosis of FA was made only after the appearance of their cancer and its treatment.[17] (some genotoxic anticancer agents are contraindicated in FA patients because of their DNA repair deficiency).The diagnosis of *FANCA* variants in these three men has also relevance in case they develop this type of cancers in the future and thanks to our investigation, they are now receiving specific medical attention including strict follow-up by oncohematologists. The link between severe

spermatogenic impairment and chronic diseases has been advocated by epidemiological observations, showing that infertile men (oligo/azoospermic) have a higher risk of morbidity (including cancer) and a lower life expectancy.[18–20] A biological basis for such observations may be: i) the previously observed high CNV burden[2] (indicating chromosomal instability) in infertile men; ii) that some genes involved in spermatogenesis may also have a role in carcinogenesis (especially those involved in stem cell renewal/differentiation and mismatch repair mechanisms[21]). Besides its clinical importance, our study stimulates further investigations on the discovery of common genetic factors involved both in SCOS and bone marrow failure.

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Disclosure:

The authors have nothing to disclose.

Table 1. Clinical characteristics of the patients carrying *FANCA* variants and of their brothers

	04-170	04-170 brother	14-339	14-339 brother
Andrological/ Endocrine and metabolic parameters				
Semen phenotype/ Testis histology	Azoospermia/ SCOS type I	Azoospermia/ not performed	Azoospermia/ SCOS type II	Normozoo-spermia
Age at first semen analysis/Testis biopsy	32	n.a	34	33
Testis volume, left;right (ml)	5; 12	n.a.	12; 10	25; 25
Relevant clinical findings	Bilateral cryptorchdism; mild dismorphic *facies*	n.a	Low stature, *cafe au lait* spots	No
FSH (IU/L)	25.6	23.3	21.3	4.54
LH (IU/L)	5.9	10.2	7.53	4.9
T (nmol/L)	15.3	8.2	20.2	18.8
Height (cm)	175	178	158	179
BMI (kg/m^2)	26.12	26.51	20.83	20.29
Glycaemia (mmol/L)	5.7	4.61	5.3	5.0
Cholesterol (mmol/L)	3.47	5.19	4.2	4.61
Hematological parameters				
Erythrocyte(10^{12}/L) *Reference Values: 4.30-6.00*	4.94	4.27	3.43	4.86
Hemoglobin (g/L) *Reference Values: 140-180*	161	136	132	145
Hematocrit (L/L) *Reference Values: 0.40-0.52*	0.48	0.40	0.38	0.43
Leucocytes (10^9/L) *Reference Values: 4.00-10.50*	4.02	3.02	3.70	5.39
Platelets (10^9/L) *Reference Values: 150-400*	194	116	137	174
MCV (fL) *Reference Values: 80-100*	98.10	92.97	112.30	89.30
MCH (pg) *Reference Value 27-34*	32.60	31.85	38.60	29.80
Neutrophils *Reference Values: 50-70*	49.30%	48.00%	49.60%	43.50%
Lymphocytes *Reference Values: 16-45*	36.10%	41.10%	32.90%	38.60%
Monocytes *Reference Values: 3-12*	9.60%	9.30%	15.90%	8.20%
Eosinophils *Reference Values: 0.40-6.60*	1.10%	1.30%	1.10%	9.10%
Basophils *Reference Values < 1.00*	0.40%	0.30%	0.50%	0.60%

SCOS type I: Sertoli Cell Only Syndrome without germ cells in **all** tubules; SCOS type II: Sertoli Cell Only Syndrome with germ cells in a minority of tubules. n.a. not available

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FIGURE LEGEND

Figure 1. Investigation on the patient 04-170 and his brother, both carriers of the FANCA variant. A) The inverted pyramid scheme indicates how WES data were filtered and prioritized for patient 04-170 starting from a total of 20477 variants. Eight homozygous, rare and potentially pathogenic variants were identified in eight different genes (in bold gene expressed in testis). B) Graphic representation of the chromosomal breakage test for patient 04-170 and his brother. The arrows illustrate the patient's and his brother's position in relation to the distribution of historical data collected in our laboratory according to the percentage of DEB-induced aberrant cells and the number of breakages in each DEB-induced aberrant cell. In the No FA group only individuals with at least one aberrant cell were included. C) The pedigree structure shows the segregation of the c.2639C>T (p.Arg880Gln) variant. Colored symbols are explained in the legend in the lower part of the figure. The lower-left arrow indicates the proband 04-170 who was subjected to WES analysis whereas his brother' DNA was sequenced by Sanger sequencing .. Our patient and his brother both carried the c.2639G>A (p.Arg880Gln) variant in homozygosis and were azoospermic. The parents' DNA samples were not available for testing, but being the variant rare it can be assumed that the parents were both heterozygous carriers

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Conflict of interest declaration:

Concenring the manuscript entitled "**From whole exome analysis in idiopathic azoospermia to the identification of a high risk subgroup for occult Fanconi Anemia"** to be considered for publication in Genetics in Medicine we declare no conflict of interest. No Pharmaceutical industry has been involved. The work in this paper has been funded by the Spanish Ministry of Health FIS awarded to Csilla Krausz (grant number: FIS/FEDER PI14/01250) and Jordi Surrallés is funded by ICREA Academia, EUROFANCOLEN project HEALTH-F5-2012-305421, CIBERER (CIBERER is an initiative of the Instituto de Investigación Carlos III) and SAF2015-64152-R/FEDER

On behalf of the authors,

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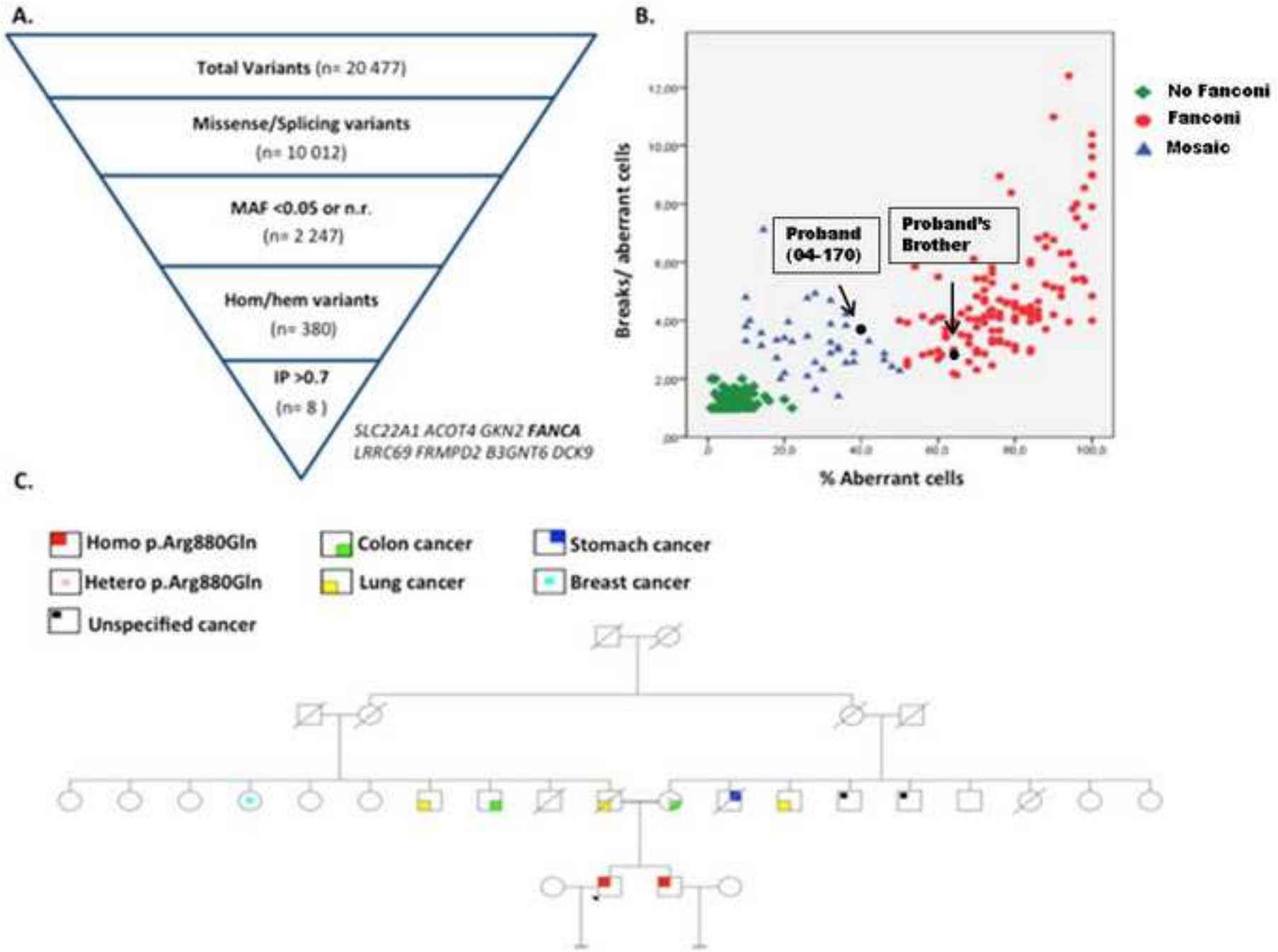
Figure 1



SUPPLEMENTARY INFORMATION

Supplement to Krausz C, Riera-Escamilla A, Chianese C et al. , **From whole exome analysis in idiopathic azoospermia to the identification of a high risk subgroup for occult Fanconi Anemia**

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Supplementary Materials and Methods

Whole-exome Sequencing

The analysis (starting from the standard DNA quality check) has been provided as SERVICE by BGI (BGI TECH SOLUTIONS (Hong Kong). The description below derives from the limited information provided by the company.

After the standard DNA quality control steps, whole- exome sequencing capture was performed using the BGI Human 59M Exon kit based on Combinatorial Probe- Anchor Ligation (cPAL™) technology[1] and the captured material was sequenced on Complete Genomics platform as service. According to the information provided by the service provider, the qualified genomic DNA was randomly fragmented to an average size of 200-400 bp and then, AdA 5'- and 3'-adaptors were ligated to the 5'- and 3'-ends of the fragments, respectively. The AdA adaptor-ligated fragments were amplified by PCR, and the PCR products were used for the follow-up exon captured (BGI exome V4, 59Mb).

During the exon capture, the exon fragments hybridized with the capture RNA 100nt kit probes stably and were therefore captured effectively. The captured exon fragments were purified by DynabeadsM-280 Streptavidin Bead purification and were further amplified by another round PCR. Then, the PCR products were circularized and the resulting double strand (ds) circles were digested with Ecop15. Among these digested fragments, small fragments were collected after bead purification. Similar to the AdA adaptor ligation, AdB were ligated to both end of the purified fragments (aforementioned). These fragments were denatured into two single strands (ss) and the target strands were selected for the circularization. The ss circles as templates were amplified to be DNA nanoballs (DNBs). DNBs were loaded on the slides and sequenced on the Complete Genomics' platform with a 100x mean coverage depth.

Bioinformatic analysis

Sequence reads were mapped to the reference genome (GRCh37) and variants were called in the aligned genome according to the methods described by Carnevali et al 2012[2] using version 2.4 of the Complete Genomics software. Briefly, exome reads were analyzed using a proprietary technology based on Teramap for alignment on GRCh37

(hg19) reference sequence. Variants with low-quality reads were excluded to guarantee ≥ 80% of bases with quality score of ≥Q20. According to the alignment results, regions of the genome deemed likely to differ from the reference genome were identified. Then, individual reads that were likely to lie in those regions were collected and a local de novo assembly was performed. Next, based on the initial mapping and assembly results, a probability statistical model (Bayesian Modeling) was applied to call variants by computing a probability ratio for any two hypotheses from the optimization step; variant calls are then made based on the most likely hypothesis according to this Bayesian probability model. Variants extracted from those hypotheses with a likelihood exceeding the significance threshold were reported.

Variants filtering

Firstly, standard filtering was applied to all samples. Briefly, we selected missense variants, stop gains/losses, frameshift insertions/deletions, and filtered out common polymorphisms (≥5% in the general population) after consulting the dbSNP 138 and the 1000G (http://www.1000genomes.org). After applying the abovementioned filter, we filtered for hemizygous and homozygous variants for which the global minor allele frequency (MAF) was ≤0.05 or not reported. Overall, obtained data was further filtered according to their potentially damaging effect depending on the type of variant. For SNVs, the prediction of pathogenicity was based on SIFT, Polyphen2, Mutation Taster, Mutation Assessor, RadialSVM, LRT and LR prediction tools. An index of pathogenicity was created as a score calculated on the basis of the seven prediction tools employed, each providing a value ranging from -1 (null probability of being a deleterious variant) to 1 (full probability of being a deleterious variant), as illustrated in Table S3. Not all prediction tools were always available; therefore a ratio was calculated between the summary score of pathogenicity and the number of prediction tools available for a determined variant (see table S3) We filtered in variants with IP>0.7 which corresponds to an arbitrary threshold, which means that the majority (not necessary all) of the interrogated tools gave a "pathogenic" score. We considered of further interest only filtered homozygous variants that were present in genes with potential implication in the early phases of spermatogenesis (Figure S1A). Concerning indels and nonsense variants prediction tools are not always available, a different approach was employed for the selection of candidate variants: all patient-specific homo/hemizygous variants with MAF≤0.01 were filtered in. For all variants, SNVs and

indels, a thorough investigation of the literature (including papers which are dealing with genes involved in azoospermia in consanguineous families) and on-line databases was performed to select variants in genes that had a potential role in the early phases of spermatogenesis. Genes for which no data on fertility were available as well as genes with no putative implication in the early stages of spermatogenesis were excluded (Figure S1B). Then the Integrative Genomics Viewer (IGV) was employed to exclude eventual false positive calls and variants prioritized as described above were validated by Sanger sequencing (Figure S1A).

Molecular characterization of testis biopsy samples

RNA was isolated from snap frozen adult testis biopsies collected from azoospermic patients with different testis histology (obstructive azoospermia with normal spermatogenesis, SCOS, spermatogenic arrest at various stages). The molecular characterization consisted in the expression analysis of four genes known to be expressed in different stages of spermatogenesis: *DAZ* (spermatogonia/early spermatocytes), *CDY1* (Spermatids), *BRDT* (pachytene spermatocytes/round and elongating spermatids) and *PRM2* (spermatids/mature spermatozoa). The housekeeping reference gene was *GAPDH*. Based on the obtained expression profiles we selected three SCOS sample, one with spermatogenic arrest predominantly at spermatogonial stage (SGA), one sample with spermatocytic arrest (SCA) and two samples with obstructive azoospermia (OA) i.e. conserved spermatogenesis including mature spermatozoa. The extraction was performed through a combination of two commercially available kits, the TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) and the AllPrep DNA/RNA kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Lifetechnologies, Foster City, CA, USA). qRT-PCR was performed using the TaqMan® Universal PCR Master Mix (Lifetechnologies, Foster City, CA, USA) with the following standard thermal cycler conditions: 40 cycles at 95 °C for 30 seconds and 60 °C for 1 min. Commercially available assays were employed to evaluate gene expression of four genes used for characterization(*BRDT, CDY1, DAZ, PRM2*) , the target gene (*FANCA*) and *GAPDH*, as reference gene for relative quantization of the target gene (Table S2).qRT-PCR runs were performed on a StepOne™ System (Applied biosystems, Carlsbad, CA, USA). Experiments were run in triplicates. Results concerning *FANCA* gene expression in the different testis biopsies are reported in Figure S4.

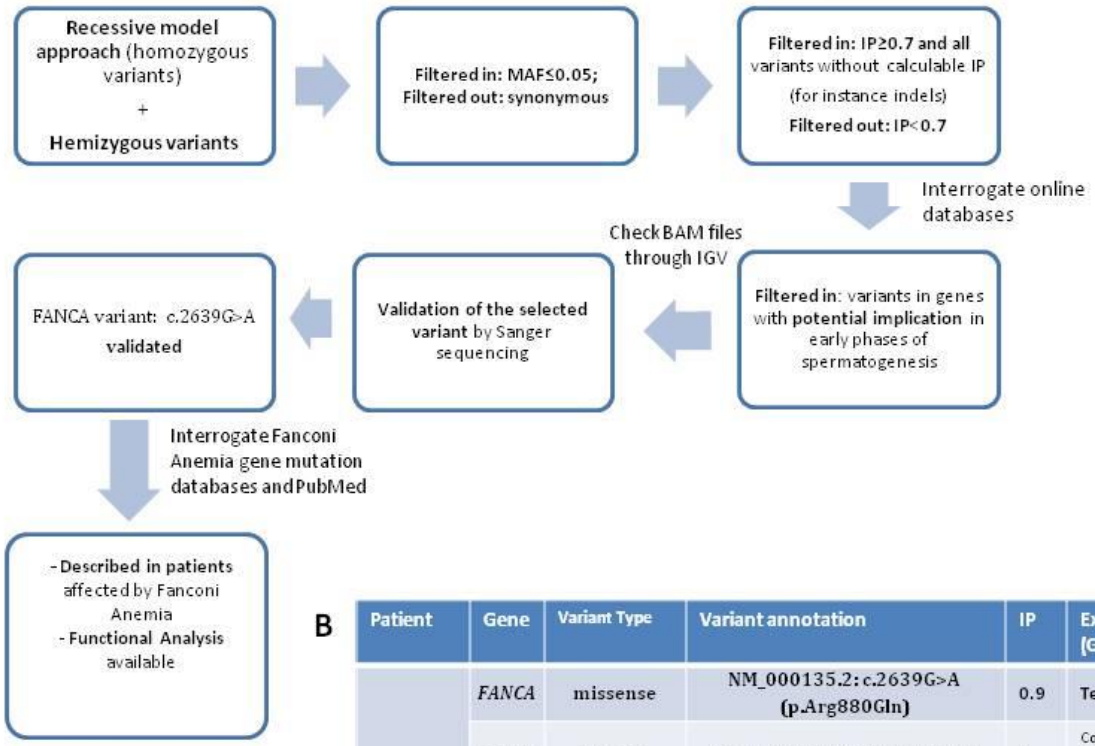
Detailed description of Diepoxibutane (DEB)-induced chromosomal breakage test

Three blood cultures were prepared for each patient, including 0.5 ml of blood in heparin and 4.5 ml of culture consisting of 15% fetal bovine serum, 1% antibiotics, 1% L-glutamine and 1% phytohaemagglutinin in RPMI (all reagents from Gibco, Carlsbad, CA, USA). Twenty four hours after culture set-up, two cultures were treated with DEB at a final concentration of 0.1 ug/ml (Sigma, Cat. No 202533, St. Louis, MO, USA), and the remaining culture was left untreated for spontaneous chromosome fragility evaluation. Forty-six hours after DEB treatment, colcemid was added at a final concentration of 0.1 ug/ml. Cultures were harvested 2 h later when metaphase spreads were obtained according to standard cytogenetic methods and finally, stained with Giemsa. For chromosome fragility evaluation, 25-50 metaphases with 46 centromeres were analyzed for each culture. The microscopic analysis was performed with a LeitzAristoplan microscope and, later with a Zeiss Imager M1 microscope coupled to a computerassisted metaphase finder (Metasystems, Altlussheim, Germany). The main criteria for the determination of chromosome fragility were as follows: gaps were not counted as chromosome breaks and figures were converted to the minimum number of breaks necessary to form each figure. DEB stock was routinely replaced every 6 months. Before using a new lot, a control fragility assay was performed using an FA lymphoblastoid cell line to ensure that there was no significant variation between lots.

<u>Figure S1</u>. Flowchart inherent to the exome analysis of patient 04-170.

The scheme briefly resumes how variants were filtered and then prioritized in patient 04-170, reaching to the identification and validation of the *FANCA* variant. A) Criteria used for filtering (after standard quality control) and prioritization; B) List of the filtered variants and their prioritization according to their expression in testis and their index of pathogenicity (IP). The only gene with testis expression is reported in bold (*FANCA*).

A



B

Patient	Gene	Variant Type	Variant annotation	IP	Expression in tissues (Genecards database)
04-170	***FANCA***	**missense**	**NM_000135.2: c.2639G>A (p.Arg880Gln)**	**0.9**	**Testis**
	B3GNT6	missense	NM_1387064: c.989T>G (p.Leu330Arg)	1	Colon-transverse, stomach, Small Intestine Terminal Ileum, and Esophagus - Mucosa
	DOCK9	missense	NM_015296.2: c.6098C>T (p.Thr2033Met)	1	Breast
	FRMPD2	splice_acceptor	NM_001018071.3:c.922G>A	1	Brain
	LRRC69	splice_donor	NM_001129890.1:c.934T>A	1	Bone marow, mesenchimal cells
	SLC22A1	missense	NM_003057.2: c.113G>A (p.Gly38Asp)	0.9	Liver
	GKN2	missense	NM_182536.2: c.193G>T (p.Asp65Tyr)	0.9	Stomach
	ACOT4	missense	NM_152331.3: c.568T>A (p.Tyr190Asn)	0.7	Liver, kidney, cortex

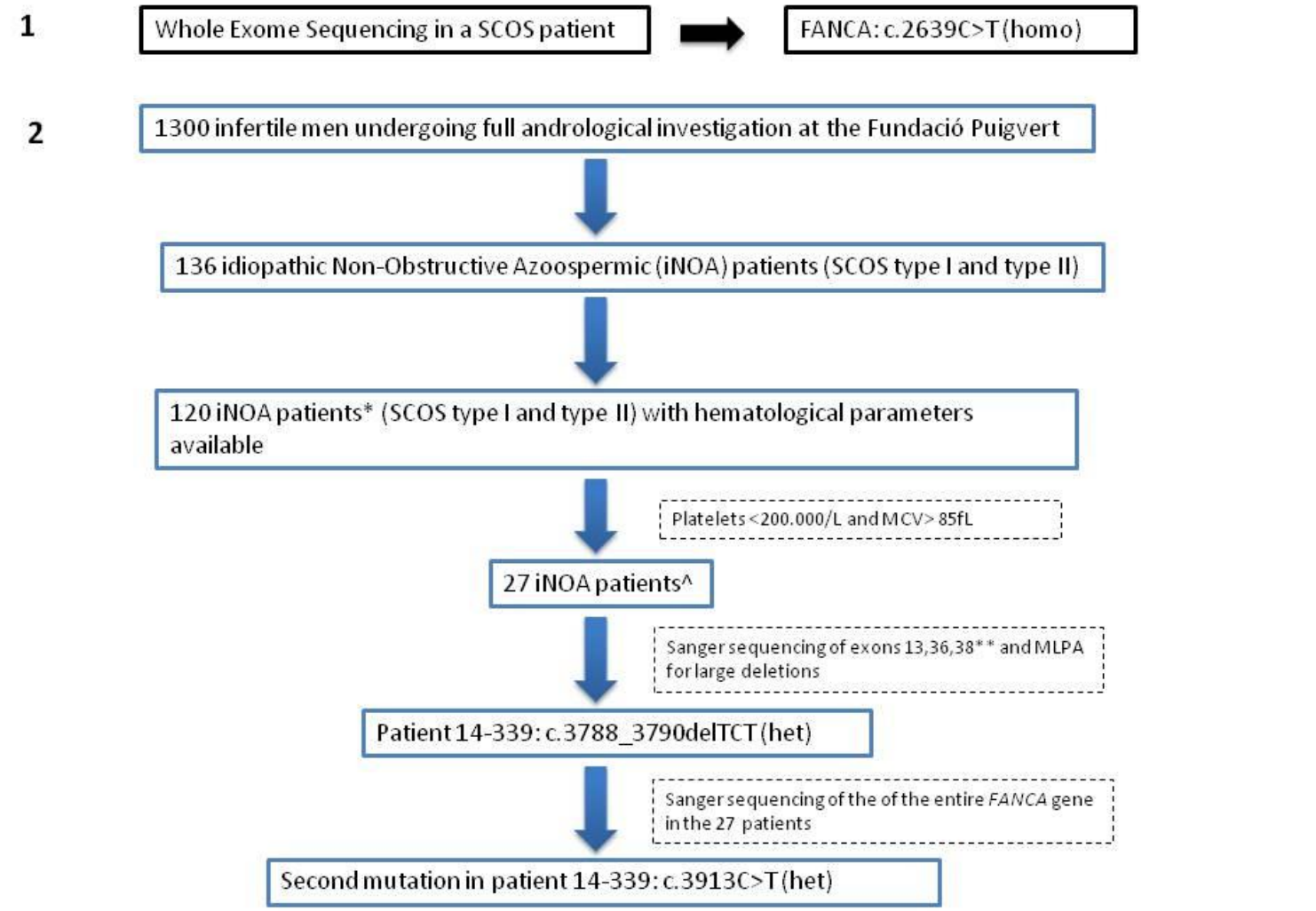
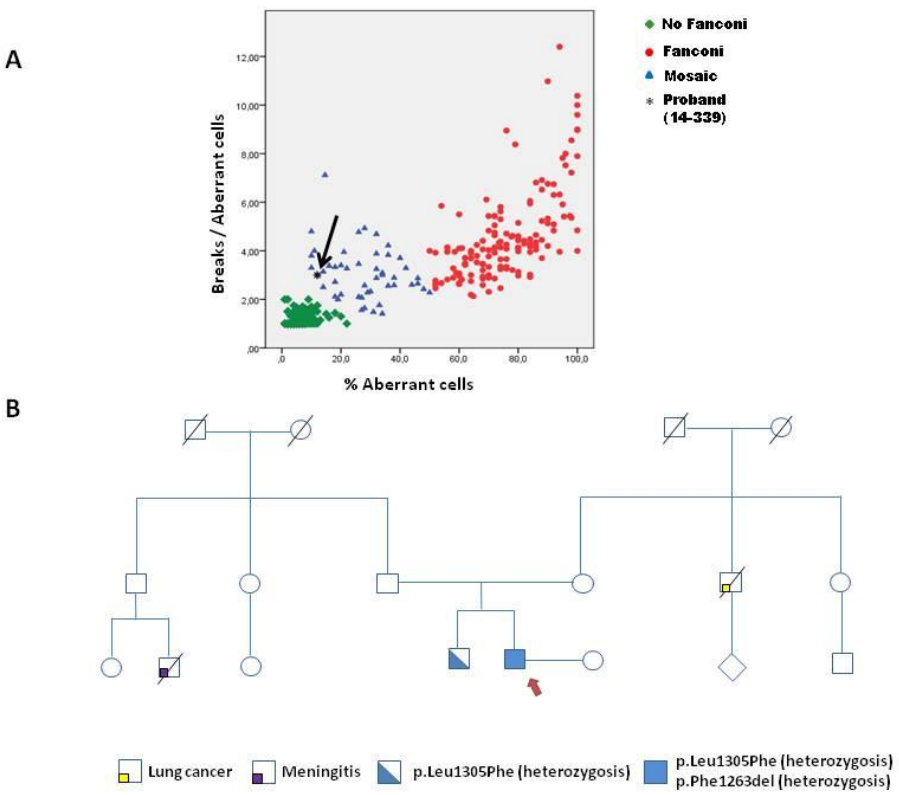


Figure S2. Flowchart reporting the study design leading to the identification of *FANCA* mutation carriers. A) The patient analyzed by Whole exome Sequencing and the mutation identified is reported. B) The scheme briefly resumes the criteria according to which infertile patients were selected and their DNA sequenced based on their testis histology, hematological parameters and genetic finding



<u>Figure S3.</u> Investigation on the patient 14-339 and his brother. Graphic representation of the chromosomal breakage test for 14-339. The graph illustrates the patient's position (*) in relation to the distribution of historical data collected in our laboratory. The pedigree structure shows the segregation of the p.Phe1263del and p.Leu1305Phe. Colored symbols are explained in the legend in the lower part of the figure. Patient 14-339 and his brother were sequenced for FANCA mutations. The lower-left arrow indicates the proband 14-339. The brother who was normozoospermic only carried the p.Leu1305Phe variant in heterozygosis. The parents' DNA samples were not available for testing, but being the variant rare it can be assumed that the parents were both heterozygous carriers

<u>Figure S4</u>. Expression evaluation of the *FANCA* gene

Quantitative RT-PCR (qRT-PCR) analysis was performed to evaluate *FANCA* expression in biopsy samples of different types of adult testis histologies: i) three SCOS (Sertoli Cell-Only Syndrome); ii) one SGA: maturation arrest at the spermatogonial level; iii) one SCA: maturation arrest at the spermatocytic level. Two samples with obstructive azoospermia (OA) were used as internal controls. Samples were first characterized by testing for four spermatogenic markers expressed at different stages of spermatogenesis: *PRM2* (spermatids/mature spermatozoa); *CDY1* (spermatids); *BRDT* (pachytene spermatocytes/round and elongating spermatids) and *DAZ* (spermatogonia/early spermatocytes)

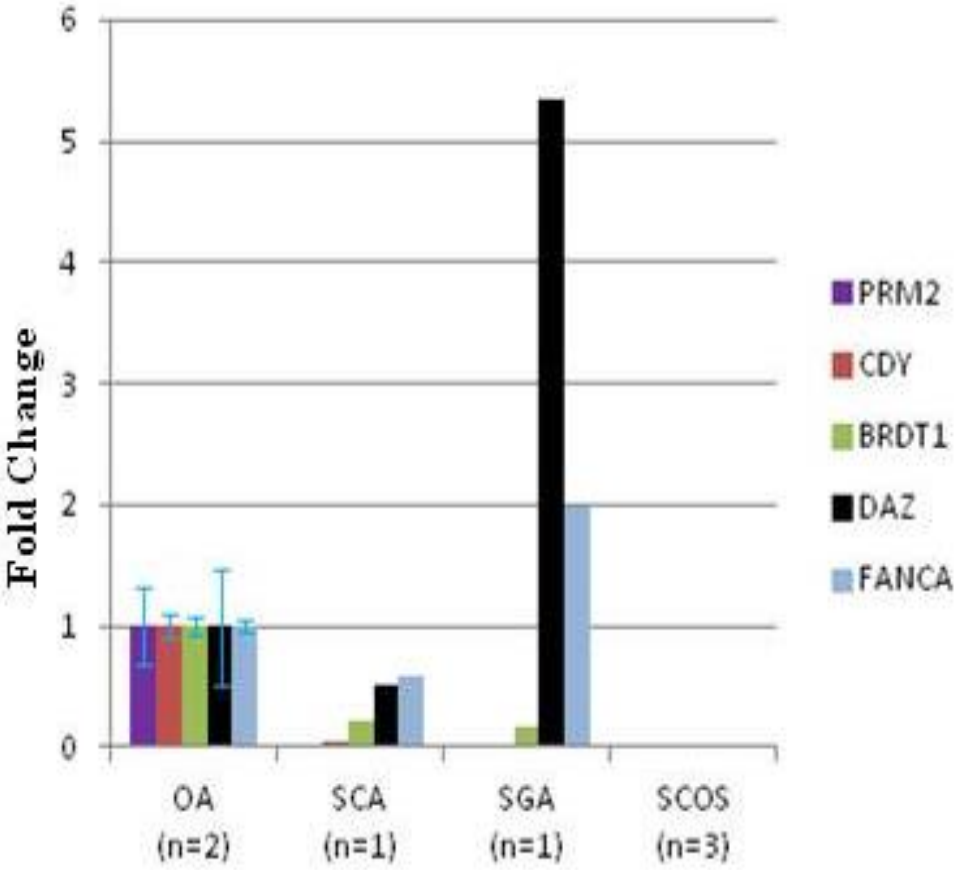


Table S1. Clinical characteristics of the 27 selected iNOA patients for *FANCA* gene mutational screening

Patient code	Semen Phenotype	Testicular phenotype	Platelets (10^9/L)	MCV (fL)	Erythrocytes (10^{12}/L)	Leucocytes (10^9/L)	Neutrophils (%)
14-339*	Azoospermia	SCOS type II	137	112.3	3.43	3.7	49.5
10-331	Azoospermia	SCOS type II	103	93.1	4.75	6	46.4
11-332	Azoospermia	SCOS type I	116	90.6	4.43	3.8	56.9
10-281	Azoospermia	SCOS type I	135	99.2	4.82	6.95	55.7
11-585	Azoospermia	SCOS type I	142	92.2	5.41	5.24	54.4
12-603	Azoospermia	SCOS type I	156	89.5	4.59	7.46	57.8
12-612	Azoospermia	SCOS type I	159	92.9	3.09	6.06	60.4
09-195	Azoospermia	SCOS type II	165	85.1	5.23	8.82	75.8
10-203	Azoospermia	SCOS type II	166	89.2	5.76	7.15	54.3
08-457	Azoospermia	SCOS type I	175	85.1	5.24	5.71	45.7
16-394	Azoospermia	SCOS type II	175	89.1	5.13	4.51	48.8
05-205	Azoospermia	SCOS type I	176	89.5	4.76	7.48	86.7
06-111	Azoospermia	SCOS type II	177	85.7	4.61	4.56	50.2
07-411	Azoospermia	SCOS type I	177	88.5	5.02	7.9	59.7
04-164	Azoospermia	SCOS type II	178	86.8	5.59	7.64	58.0
11-381	Azoospermia	SCOS type I	182	86.3	4.84	5.8	56.0
15-546	Azoospermia	SCOS type I	182	93.9	4.91	7.7	55.4
07-478	Azoospermia	SCOS type I	182	93.1	5.21	6.86	54.2
10-324	Azoospermia	SCOS type I	183	93.8	4.49	4.97	40.2
15-547	Azoospermia	SCOS type I	187	93	4.59	7.56	50.5
15-368	Azoospermia	SCOS type I	188	88.9	4.77	5.07	51.4

Patient code	Semen Phenotype	Testicular phenotype	Platelets (10^9/L)	MCV (fL)	Erythrocytes (10^{12}/L)	Leucocytes (10^9/L)	Neutrophils (%)
06-183	Azoospermia	SCOS type II	188	88.3	5.3	11.88	64.9
09-263	Azoospermia	SCOS type II	191	88.1	5.61	9.4	60.2
11-417	Azoospermia	SCOS type I	193	86.2	5.15	6.55	58.3
14-277	Azoospermia	SCOS type I	196	88.7	5.13	6.85	52.9
14-313	Azoospermia	SCOS type I	197	92.9	4.79	6.55	60.8
10-393	Azoospermia	SCOS type I	200	85.1	5.74	8.82	54.2

*Patient 14-339 carrying 2 heterozygous pathogenic mutations in *FANCA* gene. Reference value for erythrocytes: 4.3-6.0x10^{12}/L; MCV: 80-100fL; Leucocytes 4.0-10.5x10^9/L; Neutrophils: 50-70%; Platelets: 150-40010^9/L

Table S2. Commercially available TaqMan gene expression assays in testis biopsies.

Gene	Function	Assay ID
FANCA	Target gene	Hs01116668_m1
DAZ	Spermatogonia/early spermatocytes biomarker	Hs00414014_m1
CDY1	Spermatids biomarker	Hs00371514_m1
PRM2	Spermatids/mature spermatozoa biomarker	Hs04187294_g1
BRDT	Pachytene spermatocytes/round and elongating spermatids	Hs00976114_m1
GAPDH	Reference housekeeping gene	Hs0275891_g1

Table S3. Algorithms employed to calculate the index of pathogenicity for variant prioritization.

Algorithm	Prediction	Meaning	Numeric value (u)
SIFT	T	tolerated	-1
	D	damaging	1
	NA	not assigned	0
Polyphen	B	Benign	-1
	P	probably damaging	0.5
	D	damaging	1
	NA	not assigned	0
Mutation Assessor	N	neutral	-1
	L	low	-0.5
	M	medium	0.5
	H	high	1
	NA	not assigned	0
RadialSVM	T	tolerated	-1
	D	damaging	1
	NA	not assigned	0
MutationTaster	P	polymorphism	-1
	D	damaging	1
	NA	not assigned	0
LRT	N	tolerated	-1
	D	damaging	1
	NA	not assigned	0
LR	T	tolerated	-1
	D	damaging	1

Index of pathogenicity (IP) was calculated according to the following formula:

$$\frac{\left(\sum_{r=1}^{n} u_r\right)}{k}$$

Where **k**= number of available prediction tools.

Table S4. Variants detected in *FANCA* gene

Patient code	Mutation	Status	Functional study available	Previously reported
04-170 & brother	NM_000135.2: c.2639C>T (p.Arg880Gln)	Homozygosis	Yes[+]	Reported in 8 FA patients*. Described in monozygotic twins without hematological signs[+]
14-339	NM_000135.2: c.3788_3790delTCT (p.Phe1263del)	Heterozygosis	Yes[£]	Reported in 287 FA patients*.
	NM_000135.2:c.3913C>T (p.Leu1305Phe)	Heterozygosis	Yes[£]	Reported in 5 FA patients*.
14-339's brother (Not affected)	NM_000135.2:c.3913C>T (p.Leu1305Phe)	Heterozygosis	Yes[£]	Reported in 5 FA patients*.

*Fanconi anemia Mutation Database at Rockefeller University (LOVD) https://databases.lovd.nl; [+]Mankad et al. 2006[3]; [£]Castella et al. 2011[4]

Supplementary References:

1 Drmanac R, Sparks AB, Callow MJ, *et al.* Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* 2010; **327**: 78–81.

2 Carnevali P, Baccash J, Halpern AL, *et al.* Computational techniques for human genome resequencing using mated gapped reads. *J Comput Biol* 2012; **19**: 279–92.

3 Mankad A, Taniguchi T, Cox B, *et al.* Natural gene therapy in monozygotic twins with Fanconi anemia. *Blood* 2006; **107**: 3084–90.

4 Castella M, Pujol R, Callen E, *et al.* Origin, functional role, and clinical impact of Fanconi anemia FANCA mutations. *Blood* 2011; **117**: 3759–69.

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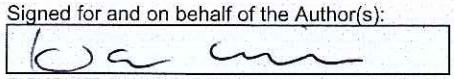
Signed for and on behalf of the Author(s): 

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