Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome

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Summary

Background About 13% of cases of non-obstructive azoospermia are caused by deletion of the azoospermia factor (*AZF*), a gene or gene complex normally located on the long arm of the Y chromosome. Oligozoospermia is far more common than azoospermia, but little is known about genetic causes. We investigated whether severe oligozoospermia is caused by *AZF* deletions and, if so, whether those deletions are present in mature spermatozoa.

Methods By PCR, we tested leucocyte DNA, from 35 men who presented at infertility clinics and who had severe oligozoospermia, for the presence of 118 DNA landmarks scattered across the Y chromosome. In the two men in whom Y-chromosome deletions in leucocyte DNA were detected, we also tested leucocyte DNA from the individuals' fathers, and in one man we tested sperm DNA.

Findings In two men with ejaculate sperm counts of 40 000–100 000 per mL, we detected Y-chromosome deletions in leucocyte DNA similar in location to those previously reported in azoospermic individuals. No Y-chromosome deletions were detected in the fathers of the two men. For one of the two men, sperm DNA was tested, and it showed the same Y-chromosome deletion seen in leucocytes.

Interpretation The Y-chromosome deletions in these two men are de-novo mutations, and are therefore the cause of their severe oligozoospermia. Not only is the absence of *AZF* compatible with spermatogenesis, albeit at reduced rate, but also the resultant sperm bear the mutant Y chromosome. Because intracytoplasmic sperm injection is increasingly used as a means of circumventing oligozoospermia, *AZF* deletions could be transmitted by this practice, and would probably result in infertile sons. In cases of severe oligozoospermia, it may be appropriate to offer Y-DNA testing and genetic counselling before starting assisted reproductive procedures.

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Introduction

Idiopathic oligozoospermia, defined as the presence of less than 10–15 million sperm per mL of semen, is the most common cause of male infertility. About 3–4% of men have severe defects in sperm production resulting in oligozoospermia, a principal or contributing factor in up to a fifth of infertile couples.[1,2] Progress towards medical therapies to correct oligozoospermia has been slow, at least partly because the aetiology of the disorder is not understood. In particular, little is known about the possible contributions of genetic factors.

A contrast is provided by cases of azoospermia caused by Y-chromosome deletions. In 1976, Tiepolo and Zuffardi[3] reported the occurrence of grossly deleted Y chromosomes in six azoospermic men, and on this basis they postulated the existence of a Y-borne gene or gene complex required for spermatogenesis. We have shown this hypothesis to be correct, and azoospermia factor (*AZF*) has been localised precisely. About 13% of men with non-obstructive azoospermia have deletions of a particular and consistent region of the long arm of the Y chromosome.[4] Such findings alone were insufficient to prove that the Y-chromosome deletions were the cause of azoospermia in these men; many Y-DNA sequence variants, even those involving apparent deletions, are polymorphisms of little or no functional consequence, readily passed from one generation to the next.[5] Causality could be inferred only if the Y-chromosome variants in the infertile men were shown to be new mutations—ie, not present in their fathers—from whom they inherited their Y chromosomes.[6] In the azoospermic men described above, we found that the Y-chromosome deletions were not present in the affected individuals' fathers but had arisen de novo. This finding established unequivocally that the deletions were the cause of azoospermia in those men.[4]

Examination of testis biopsy samples from azoospermic men with *AZF* deletions revealed substantial histological variation. In several cases, germ cells were absent, and a histological diagnosis of Sertoli-cell-only syndrome could be made. In other cases, early spermatogenic cells were observed, leading to a histological diagnosis of testicular maturation arrest. In two of the latter samples, spermatogenesis had sporadically progressed to the condensed spermatids stage within each sample. These results suggested that, at least when associated with Y-chromosome deletions, Sertoli-cell-only syndrome and testicular maturation arrest share a common cause—the loss of the *AZF*.[4]

The range of testicular histology observed in azoospermic men with *AZF* deletions led us to postulate that spermatogenic defects less severe than azoospermia are also caused by the absence of *AZF*. What if the *AZF* is not absolutely necessary for completion of spermatogenesis? Although the *AZF* gene or gene complex was originally defined and mapped in the context of azoospermia, we wondered whether the gene's absence might sometimes result in oligozoospermia. A few cases of Y-chromosomal variants have been reported in

STS	Gene	Left primer	Right primer	Product size (bp)
sY277	*DAZ*	GGGTTTTGCCTGCATACGTAATTA	CCTAAAAGCAATTCTAAACCTCCAG	275
sY279	*DAZ*	CCACCTCATGGTAGTAAAATTGTA	CTCTTATTTATCTTATTGCTACAACG	150
sY283	*DAZ*	CAGTGATACACTCGGACTTGTGTA	GTTATTTGAAAAGCTACACGGG	375
sY274	*RPS4Y*	TTAAGGGGACAGTATTTCAACTTC	CCACATTTAAACTGAGTACAGTCC	350
sY238	*ZFY*	AACAAGTGAGTTCCACAGGG	GCAAAGCAGCATTCAAAACA	350
sY276	*AMELY*	CCTACCGCATCAGTGAATTTC	TCTGTATGTGGAGTACACATGG	200

Table: **Designations and oligonucleotide primer sequences of six new STSs**

oligozoospermic men,[7] but no case before this study was shown to be the result of a de-novo mutation, and thus no causal link could be established. In searching for such definitive evidence of causality, we chose to focus our initial studies on men with severe oligozoospermia.

Methods

On cytology, the Y chromosome consists of a euchromatic region and a heterochromatic region. Since the heterochromatin does not affect fertility,[8,9] we concentrated on the euchromatin, for which a map of DNA landmarks and overlapping recombinant DNA clones has been constructed.[10,11]

To screen for Y-chromosome deletions by PCR, genomic DNA was prepared from peripheral blood samples[12] from 35 men who presented to infertility clinics and who had total motile sperm counts (per ejaculate) of 40 000–1 000 000. We tested each man for the presence of 118 Y-DNA landmarks (sequence-tagged sites [STSs]) previously shown[4,10,11] to encompass the euchromatic region of the Y chromosome. We took two precautions to keep false-negative results to a minimum: we only used PCR assays that reliably gave positive results when tested on 90 fertile men; and we did not record an STS as absent from a patient unless at least three successive PCR amplifications yielded negative results.

In patient WHT2712, spermatozoa were purified from ejaculate by centrifugation on a MiniPercoll gradient.[13] To prepare DNA, 50 000 sperm were incubated for 1 h at 37°C in 20 mL of a solution containing 0·05 mg/mL proteinase K, 50 mmol/L potassium chloride, 10 mmol/L Tris HCL pH 8·3, 1·5 mmol/L magnesium chloride, 20 mmol/L dithiothreitol, and 1·7 mmol/L sodium dodecyl sulphate. Proteinase K was then inactivated by heating at 85°C for 5 min, and 100 mL of PCR mix (containing 1·5 mmol/L chloride magnesium, 5 mmol/L ammonium chloride, 10 mmol/L Tris HCL (pH 8·2), 50 mmol/L potassium chloride, and 100 mmol/L deoxyribonucleotide phosphate was added to each sample.[14] This sperm DNA was tested for a subset of the Y-chromosomal STSs, again by PCR.

Most of the Y-chromosome STSs, as well as the PCR and electrophoresis conditions used, have been described previously.[4,10] Six new STSs were developed for this study (table).

Results

We detected Y chromosome deletions in two men, both of whom were severely oligozoospermic and had poor sperm motility and morphology. In the first man, WHT2615, repeated semen analyses yielded sperm counts of 50 000 to 100 000 per mL, with 20–30% of sperm motile and only 10% of sperm with normal morphology. In the second man, WHT2712, sperm counts ranged from 40 000 to 90 000 per mL, with 30–40% of sperm motile and 10–25% of sperm with normal morphology. Apart from infertility, these two unrelated men were in good health.

We discovered deletions of small, interstitial portions of the long arm of the Y chromosome (Yq), in both WHT2615 and WHT2712. In both men we detected the presence of the bulk of the Y chromosome, including the sex-determining region (SRY), Y-RNA-recognition motif genes (YRRM1 and YRRM2), the centromere, and the heterochromatic region. However, 43 of the 118 Y-chromosomal STSs were absent from the DNA of WHT2615; 44 were absent from the DNA of WHT2712. The absent STSs were all clustered in the *AZF* region, the portion of Yq commonly deleted in azoospermic men, and they include the deleted in azoospermia *(DAZ)* gene, proposed as a candidate gene for *AZF*.[4] The Yq deletions observed in the two oligozoospermic men overlap substantially not only with each other but also with the deletions we previously observed in azoospermic men (figure 1).

If the Yq deletions were the true cause of severe oligozoospermia in WHT2615 and WHT2712, the deletions should represent new mutations not present in their fathers. Both fathers were found to carry intact Y chromosomes. We conclude that the deletions of the *AZF* region are the cause of oligozoospermia in these two men.

The Y-DNA tests described above were all done on blood, a conventional and readily accessible source of DNA for genetic testing. However, our finding of *AZF*-region deletions in leucocytes from oligozoospermic men raised questions about the DNA of their sperm. If *AZF* is absolutely required for completion of spermatogenesis, the sperm produced by the two oligozoospermic men in our study could carry either an X or an intact Y chromosome, but never an *AZF*-deleted Y chromosome. This arrangement would be possible if the two oligozoospermic men were testicular mosaics for a cell line with an intact Y chromosome, in which case the de-novo *AZF* deletions reported would have arisen as somatic mutations, after fertilisation, rather than in the fathers' germlines. On the other hand, if these oligozoospermic men produce some sperm carrying *AZF*-deleted Y chromosomes, issues of genetic counselling would arise, because efforts to father children by artificial fertilisation techniques might propagate the Y-chromosomal defect and the infertility it causes.

To test these possibilities, we carried out DNA tests on the sperm produced by one of the men (WHT2712). We tested sperm-DNA prepared from an unrelated fertile man as a control. We tested for a subset of Y-chromosomal STSs located within and just outside the region deleted in the leucocytes of WHT2712. As expected, the fertile man's sperm carried an intact Y chromosome. The oligozoospermic man, WHT2712, was also found to produce Y-bearing sperm, but these carried a deletion in the *AZF* region—the same deletion as was found in his blood (figure 2).

Discussion

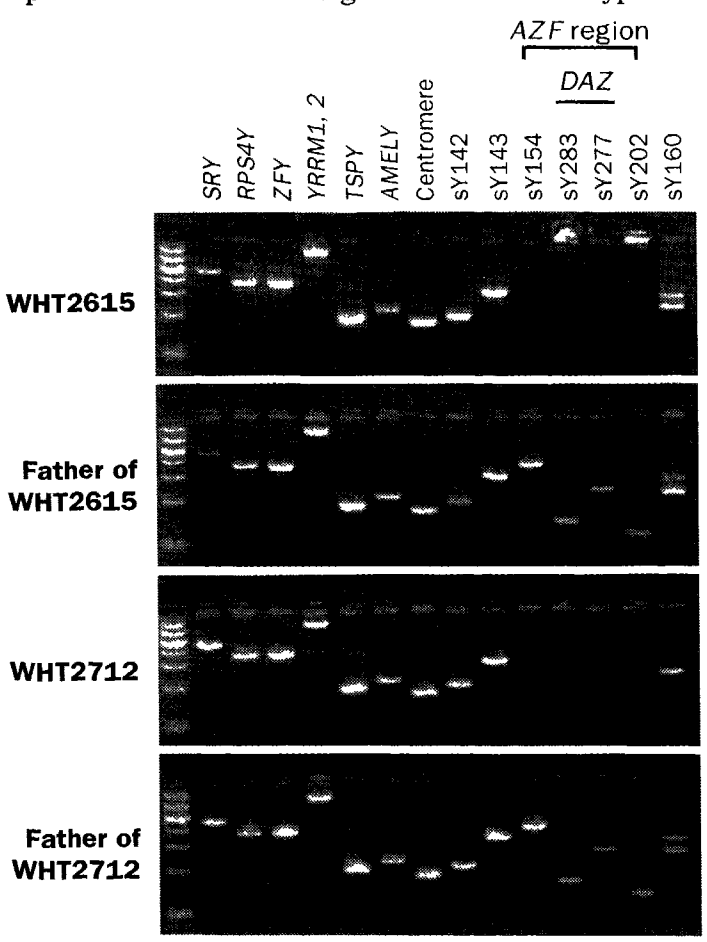
We found that severe oligozoospermia is caused, in some cases, by newly arisen deletions on the Y chromosome. These deletions are of interstitial, submicroscopic portions of Yq, and they encompass the entirety of the previously defined *AZF* region. The observation that these portions of DNA were present in the fathers of the oligozoospermic men is crucial because it establishes that the deletions were not inconsequential polymorphisms,[5] but were the cause of oligozoospermia. The Yq deletions

in the two oligozoospermic men were similar in location and extent to those we reported[4] in azoospermic men with Sertoli-cell-only syndrome or complete testicular maturation arrest (figure 1).

How could similar Yq deletions cause such an array of spermatogenic disorders? There are two possible explanations.[4] According to the first hypothesis, the nature of the spermatogenic disorders is determined solely by the deletion in the *AZF* region, and the severity of the disorder in any given individual depends on which gene or genes in the *AZF* cluster are deleted. In this hypothesis, the *AZF* must consist of at least two spermatogenesis genes. This hypothesis would predict that the severity of the spermatogenic defect was directly correlated with the extent of the Y-chromosome deletion. Our data do not support such a correlation. On the contrary, the deletions reported in oligozoospermic men are as large or even larger than those observed in some azoospermic men with Sertoli-cell-only syndrome (figure 1).

We have previously argued[4] in support of a second hypothesis and, with the inclusion of our findings in men with severe oligozoospermia, we continue to favour it. According to this explanation, environmental or random factors or genes outside the *AZF* region modify the effects of *AZF* deletions. In this instance, *AZF* could consist of one or more genes. Our deletion studies are consistent with this hypothesis. Most importantly, this model implies that severe oligozoospermia, complete testicular maturation arrest, and Sertoli-cell-only syndrome are not aetiologically distinct, at least when associated with Yq deletions, but represent clinically diverse manifestations of the same underlying genetic anomaly.

At present, we cannot estimate accurately the proportion of cases of oligozoospermia caused by *AZF* deletion or mutation. Our sample of 35 men was small and heavily skewed towards very severe oligozoospermia (sperm counts below 1 000 000 per mL). Some men in whom we failed to detect *AZF*-region deletions might have small deletions or point mutations not detectable with the DNA probes available. Two of Kobayashi and colleagues'



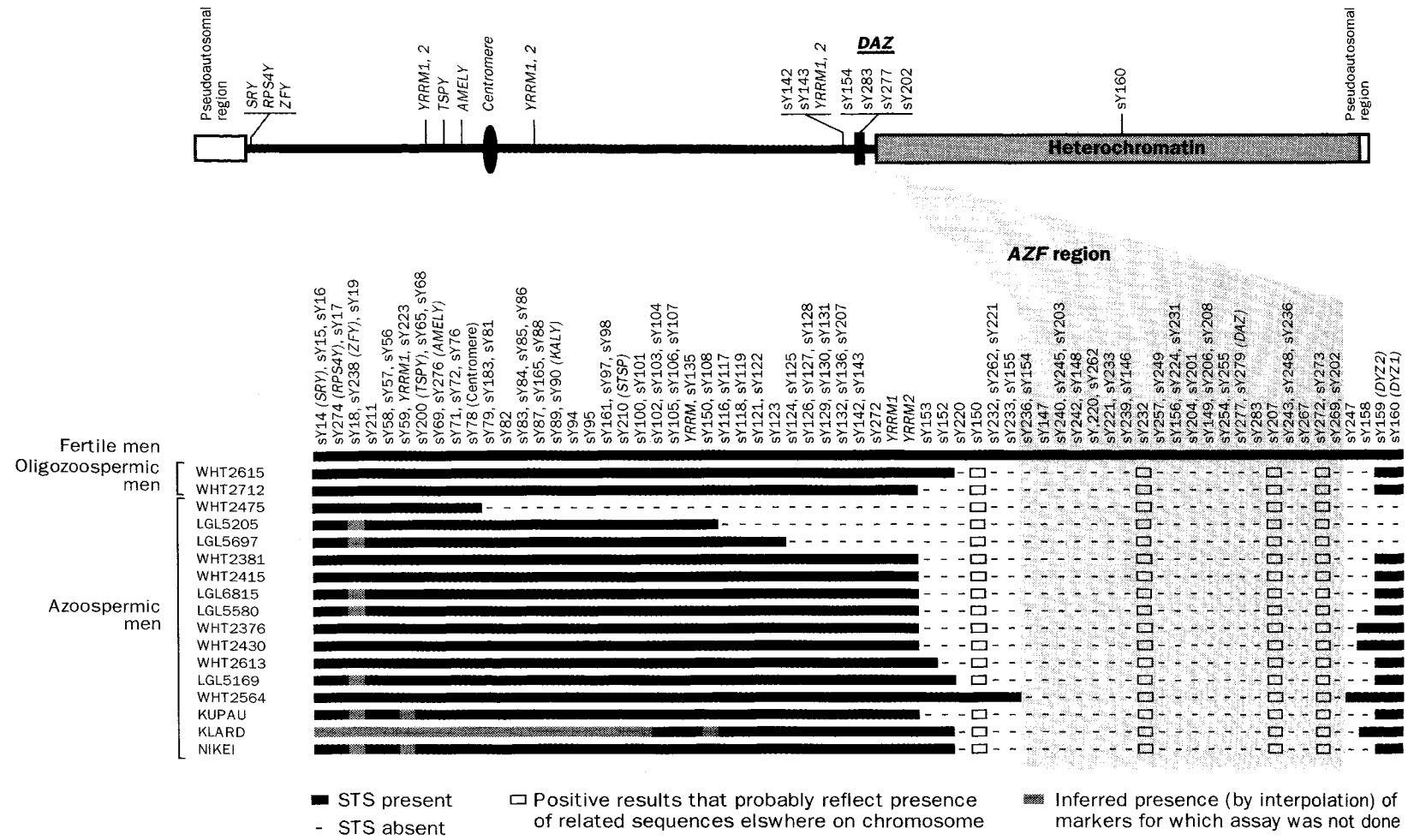


Figure 1: **De-novo deletions of *AZF* region of Y chromosome in two oligozoospermic men**
PCR products obtained from leucocyte DNAs of two oligozoospermic men (WHT2615 and WHT2712) and their fathers (top panel). Genes and landmarks indicated on diagram of Y chromosome (middle panel). Results of all Y-chromosomal markers tested on oligozoospermic men WHT2615 and WHT2712 (bottom panel). Y-chromosomal STSs (gene and locus names in parentheses) are listed on top border in order previously reported.[4,10] Results for fertile and azoospermic men shown for comparison are reported previously.[4]

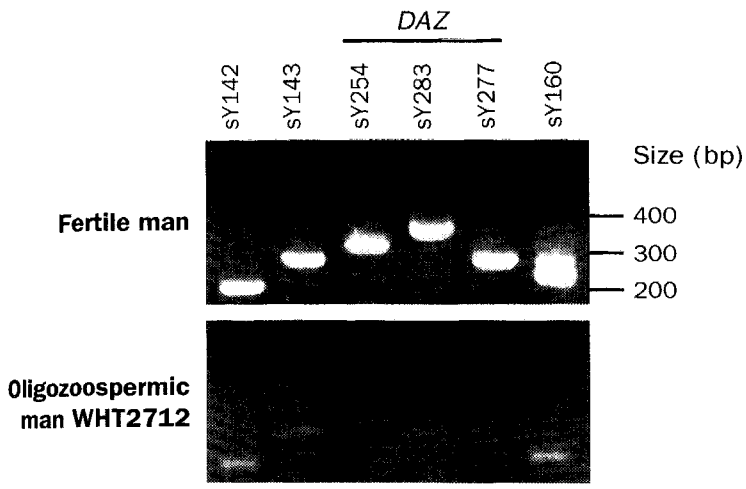


Figure 2: ***AZF*-deleted, Y-bearing sperm in semen of oligozoospermic man WHT2712**

PCR products were from DNA prepared from purified sperm. All markers tested are present in sperm from a fertile man, but *AZF* region markers sY254, sY277, and sY283 are absent in sperm from WHT2712.

oligozoospermic Japanese patients (numbers 1503 and 1958; sperm counts of 1000 and 200 000, respectively) had variant Y chromosomes,[7] and these variants may resemble the de-novo *AZF*-region deletions we describe. However, since the fathers of the two Japanese men were not studied, the functional significance of their Y variants remains unclear. A small set of Y-DNA markers was used to study the Japanese men; the results of the two sets of patients therefore cannot be compared in detail.

Our studies show that *AZF* is not strictly required for completion of spermatogenesis. Indeed, at least one of the men in whom we detected *AZF* deletions (in leucocytes) produced Y-bearing, *AZF*-deleted spermatozoa. We had previously suggested[4] that *AZF* normally influences the fate of spermatogonial stem cells or facilitates the differentiation of their precursors, the primordial germ cells. The present findings are consistent with, but do not provide definitive evidence for, this hypothesis; it would be premature to discount the possibility that *AZF* has a function at a later stage in spermatogenesis.

AZF is either a single gene or multiple genes in proximity, and the gene (or genes) and the encoded protein (or proteins) are not yet known. The *DAZ* gene, which appears to encode an RNA-binding protein, is a promising candidate gene for *AZF*.[4] *DAZ* is absent in both oligozoospermic men in whom we detected Yq deletions (figure 1). If the absence of *DAZ* is the cause of oligozoospermia in these two men, one might expect that point mutations in *DAZ* could also cause oligozoospermia. The finding of such point mutations in oligozoospermic or azoospermic men could prove that *DAZ* is *AZF*. A search for such mutations should be undertaken.

Increasing use is made of assisted reproductive techniques to circumvent the infertility or subfertility associated with oligozoospermia. Particularly encouraging is the 35% pregnancy rate that has been achieved with intracytoplasmic sperm injection.[15] Such efforts might, in theory, result in transmission to offspring of the genetic defect that caused spermatogenic failure.[16] However, no such genetic defect has been identified. The risk up until now has been hypothetical, and no specific genetic test has been indicated. This may no longer be the case for men with severe oligozoospermia, a small fraction of whom have a deletion of the Y chromosome's *AZF* region—not only in somatic cells (eg, leucocytes), but also in mature spermatozoa (figure 2). We predict that all sons fathered by men with *AZF* deletions, with the aid of assisted reproduction techniques, would themselves have *AZF* deletions. Like their fathers, these sons with *AZF* deletions would be expected to have severe spermatogenic defects but be otherwise healthy.

The majority of intracytoplasmic sperm injection procedures are for cases of oligozoospermia. However, the procedure can be used to introduce spermatids or sperm retrieved by biopsy from the testes of azoospermic men with testicular maturation arrest.[17,18] Thus, the fathers with *AZF* deletions might be either oligozoospermic or azoospermic. Given our findings in *AZF*-deleted sperm from men with oligozoospermia, we would expect the *AZF*-deleted germ cells of men with azoospermia also to carry defective Y chromosomes. In cases of severe oligozoospermia and non-obstructive azoospermia, physicians and couples may want to consider genetic counselling and Y-DNA testing before intracytoplasmic sperm injection.

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