

Reduced Human Germ Cell-Less (*HGCL*) Expression in Azoospermic Men With Severe Germinal Cell Impairment

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ABSTRACT: Germ cell-less (GCL) protein is a nuclear envelope protein highly conserved between the mammalian and *Drosophila* orthologues. In *Drosophila*, maternal GCL protein is required to establish the germ lineage during embryonic development. In mammals, it is suggested that the GCL function is mainly in spermatogenesis and that it might be related to the ability of mouse GCL to repress transcription. Using reverse transcriptase–polymerase chain reaction analyses, we investigated the role of human GCL (*HGCL*) in spermatogenesis by studying its expression in the testicular tissue of 67 azoospermic men with normal karyotype and no Y-chromosome microdeletion. Their testicular biopsy specimens underwent meticulous histological and cytological analysis as well as molecular analysis with various markers of spermatogenesis (*RBM1*, *DAZ*, and *CDY1*). The rate of X-Y and 18 chromosome bivalent formation dur-

ing meiosis was additionally assessed in 22 of these biopsy specimens and correlated to *HGCL* expression. Expression of *HGCL* was affected in parallel with the severity of testicular impairment found. Defective sperm motility was associated with the absence of *HGCL*. Nevertheless, the absence of *HGCL* expression did not influence the normal process of chromosome bivalent formation in meiosis. Our results suggest that *HGCL* is not essential for the chromosomal events of meiosis but might be involved in later aspects of spermatogenesis.

Key words: Testicular *HGCL* expression, markers of spermatogenesis, spermatogenesis impairments, motility impairments, *HGCL* and chromosome bivalent formation.

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Impairment in fertility affects 1 in 25 men (Cram et al, 2001), and the underlying causal factors are unknown in most of them. It is currently believed that idiopathic infertility is mainly of genetic origin. Thus, the study of genes that are involved in the control of spermatogenesis may help to elucidate and distinguish between intrinsic and acquired male infertility.

One gene with a potential role in human fertility is germ cell-less (*GCL*). The GCL protein was first described in *Drosophila melanogaster* as a crucial factor in embryonic germ cell development (Jongens et al, 1992, 1994). It was shown that *Drosophila* females with reduced GCL function give rise to sterile adult progeny that

lack germ cells. *Drosophila* with a Δgcl genotype is associated with impaired spermatogenesis due to the failure to establish transcription quiescence necessary for the proper formation of germ cell precursors (Robertson et al, 1999; Leatherman et al, 2002).

Mouse and human orthologues of *Drosophila* GCL (*mGCL*, *HGCL*, and *dGCL*, respectively) were recently cloned and characterized (de la Luna et al, 1999; Kimura et al, 1999; Nili et al, 2001a). An important feature of all GCL proteins is the presence of an evolutionary conserved BTB/POZ (broad-complex, tram track, and bric-a-brac/poxvirus and zinc finger) domain. This domain is an evolutionarily conserved protein-protein interaction domain often found in developmentally regulated genes (Godt et al, 1993; Bardwell and Treisman, 1994; Zollman et al, 1994). It is strongly implicated in the regulation of gene expression through oligomerization and interaction with cofactors, ultimately leading to chromatin remodeling and changes in gene expression (review by Collins et al, 2001).

mGCL was found to be expressed in low levels at the primordial germ cells and highly expressed in the adult mouse, mainly at the pachytene spermatocyte stage (Ki-

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mura et al, 1999; Leatherman et al, 2000). This protein rescued the *dGCL* null phenotype, indicating that *mGCL* is a functional orthologue of *dGCL* (Leatherman et al, 2000). *mGCL* was demonstrated to interact with the DP3 α component of the E2F-DP heterodimer transcription factor, an interaction that was found to repress the transcriptional activity of the E2F complex. This repression is thought to be mediated through anchoring of the E2F complex to the nuclear envelope, possibly through LAP2 β , a nuclear envelope protein that also binds GCL (de la Luna et al, 1999; Nili et al, 2001b). Furthermore, overexpression of *mGCL* was suggested to cause the accumulation of cells in the G1 phase, suggesting that it has properties of a negative cell-cycle regulator (de la Luna et al, 1999).

The *HGCL* gene was recently isolated and mapped to chromosome 2p13. *HGCL* expression is not ubiquitous, and the highest levels of messenger RNA were detected in the testis (Nili et al, 2001a).

Given the involvement of *mGCL* in spermatogenesis, the present study focused on the role of *HGCL* in human spermatogenesis and, particularly, at meiosis. The expression of the *HGCL* gene in testicular biopsy specimens of azoospermic men who were grouped according to their histologic and cytologic findings was evaluated. To overcome the nonhomogeneous nature of the testis, expression of *HGCL* was also correlated to that of germ cell-specific genes (*RBM*, *DAZ*, and *CDY1*), denoting the presence of germ cells in the biopsy specimen used for RNA extraction. In addition, the expression of *HGCL* was correlated to meiosis normality as measured by the rate of meiotic bivalent formation in spermatocytes.

Materials and Methods

A total of 67 azoospermic men undergoing the testicular sperm extraction procedure consented to a genetic evaluation. Only men with a normal karyotype and no Y-chromosome azoospermia factor (AZF) microdeletion were included. The institutional review board committee approved the study in accordance with the Helsinki Declaration of 1975.

Testicular Tissue Evaluation

One biopsy specimen from each testis was divided into 3 pieces: 2 small pieces (approximately 5 mg each) were taken, one for histopathological analysis and the other for RNA isolation. The third portion of the biopsy (approximately 50 mg) was minced for spermatozoa isolation to be used in the intracytoplasmic sperm injection (ICSI) process. In 22 cases, an additional small piece was minced, fixed, and further analyzed by fluorescence in situ hybridization (FISH) for meiosis evaluation (Yogev et al, 2000). Two additional biopsy specimens from other locations were taken from each testis for spermatozoa extraction only in almost all cases (Hauser et al, 1998).

Quantitative Analysis and Classification of Testicular Biopsy Specimens

Histological examination was performed on Bouin's fixed paraffin-embedded biopsy specimens after staining with hematoxylin-eosin. The most advanced spermatogenic cell that was identified determined the histological definition. The terms *mature spermatid* and *spermatozoa* were used for histological and minced biopsy specimen detection, respectively. Normal spermatogenesis in azoospermic men was classified by the mean number of mature spermatids per tubule according to the classification proposed by Silber et al (1997). At least 20 seminiferous tubules were scored in each specimen. The presence of spermatozoa in minced testicular tissue was assessed as previously described (Ben-Yosef et al, 1999). Sperm motility was qualitatively judged in each biopsy specimen immediately after mincing and at 30-minute intervals of incubation up to 2 hours.

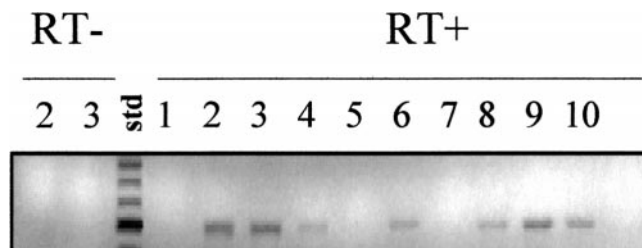
Four groups were established according to the combined findings of histological and cytological examination of the 3 biopsy specimens taken from the respective testis. The control group included 19 biopsy specimens from men with a normal number of mature spermatids per tubule (>17 sperm cells per tubule; Silber et al, 1997) by histological analysis. Fourteen of them were from male carriers of cystic fibrosis mutations/5T polymorphism combined with congenital absence of the vas deferens. The hypospermatogenesis group included 23 men in which at least one biopsy specimen contained spermatozoa or mature spermatids. The spermatocyte maturation arrest group (10 biopsy specimens) was characterized by the absence of spermatozoa or mature spermatids in all locations of both testes. The Sertoli cells only group included 15 men in which an absolute absence of germ cells was found in all biopsy specimens.

The mean \pm SE follicular stimulating hormone values in the normal spermatogenesis, hypospermatogenesis, spermatocyte maturation arrest, and Sertoli cell only groups were 5.7 ± 1.4 , 19.24 ± 2.6 , 16.3 ± 3.3 , and 20 ± 2 mIU/mL, respectively.

Genetic Evaluation

Karyotype and Y-chromosome microdeletion tests were performed on peripheral lymphocytes. Chromosome analysis was performed by the G-banding chromosome staining technique. The Y-chromosome AZF microdeletion test was performed by multiplex polymerase chain reaction (PCR) with genomic DNA as previously described (Kleiman et al, 1999).

Testicular biopsy specimens from one testis (left or right) of each man was frozen in liquid nitrogen immediately after having been dissected and cryopreserved until RNA isolation. Total RNA was extracted after homogenization by the High Pure RNA Tissue kit (Roche, Mannheim, Germany), and 10 μ L of the extract was used for complementary DNA (cDNA) synthesis with AMV reverse transcriptase (Roche) and oligo-dT₁₅. The oligonucleotide primer sets for *CDY1* minor, *DAZ*, *RBM*, and β -*actin* were previously described (Kleiman et al, 2001). The oligonucleotide primers set for the detection of *HGCL* expression (GCL-up CTATTACACATCAGCAGGGAC and GCL-down CTTGAGGCCACCTCACTGTCC) were designed from the published sequence accession XM.031592. These primer sets for *HGCL*, *CDY1* minor, *DAZ*, and *RBM* gave differential PCR products for cDNA and genomic DNA. The same PCR product



Human germ cell-less (*HGCL*) messenger RNA expression in testicular biopsy specimens analyzed by reverse transcriptase–polymerase chain reaction. 1 indicates blank; 2 and 3, normal spermatogenesis; 4 and 5, Sertoli cells only; 6 and 7, spermatocyte maturation arrest; 8 to 10, hypospermatogenesis; +RT, mixture with reverse transcriptase; –RT, mixture without reverse transcriptase; and std, 100-bp ladder standard.

for cDNA and genomic DNA was obtained with β -actin primers. In view of this observation, RNA samples were tested with and without the reverse transcriptase (RT) step to verify that there was no genomic DNA contamination. The expression of β -actin was evaluated as an internal control for the quality of the RNA isolation and efficiency of the RT-PCR method. A positive control (cDNA from the castrated man) and blank controls were included in each PCR run. Whenever PCR results were negative in 2 independent reactions, RT and PCR steps were redone.

Meiotic Evaluation

Spermatocyte evaluation with triple-color FISH analysis using centromeric DNA probes for chromosomes X, Y, and 18 (Vysis, Downers Grove, Ill) was performed (Yogev et al, 2000). The rate of bivalent formation was scored as previously described (Yogev et al, 2002). Generally, at least 350 primary spermatocytes were analyzed in each specimen, according to statistical calculations for the required sample size. The FISH technique used for bivalent evaluation had certain advantages over other techniques mainly because it facilitates the evaluation of meiosis, even in pathological cases with an extremely low number of spermatocytes that reflect serious testicular damage (Metzler-Guillermain and Guichaoua, 2000).

Statistical Analysis

The association between the groups with various impairments of spermatogenesis (determined by the combined results of the histological and cytological evaluations) and the presence of *HGCL* expression was checked using the Pearson χ^2 test. Fisher's exact test was performed to assess the significance of the relationship between the presence of expression of *HGCL* and the spermatogenic impairment.

The significance of *HGCL* association to the overall expression of *DAZ*, *RBM*, and *CDY1* and to the normal motility of sperm cells was assessed by the same test. Pearson's test was performed to assess the correlation between percentage of biopsy specimens with the spermatozoa and chromosomes bivalent formation rate. The difference in the percentage of chromosome bivalents formation between groups expressing and not expressing *HGCL* was calculated by the *t* test.

Results

RNA Expression Analysis of *HGCL* in Testicular Biopsy Specimens With Various Impairments of Spermatogenesis

Since *HGCL* is suggested to play a role in spermatogenesis, the correlation between its expression and various spermatogenic impairments was assessed. The RT-PCR results in the designated groups (ie, normal spermatogenesis, hypospermatogenesis, spermatocyte maturation arrest, and Sertoli cell only) are shown in the Figure and are summarized in Table 1. The expression of *HGCL* tested by RT-PCR was detected at variable ratios in the different groups (Table 1). A significant association between certain groups and expression of *HGCL* was found ($P = .026$). *HGCL* was expressed at a significantly higher rate in specimens in which spermatozoa were detected (normal and hypospermatogenesis groups) compared with specimens with no spermatozoa ($P = .032$). *HGCL* expression was also detected at higher rate in specimens containing germ cells (normal, hypospermatogenesis, and maturation arrest groups) compared with those with depleted germ cells (Sertoli cell only group) ($P = .029$). The most significant difference was between groups with normal spermatogenesis vs Sertoli (somatic) cell only ($P = .003$). The association between either germ cell (*DAZ*, *RBM*) or haploid germ cell (*CDY1*) molecular markers and the *HGCL* expression was significant ($P = .021$, $P = .021$, and $P = .004$, respectively).

Characteristics of Biopsy Specimens With Impairment of *HGCL* Expression

Specimens with hypospermatogenesis showed significant differences in the sperm motility between *HGCL*-express-

Table 1. Gene expression results of testicular biopsy specimens with varied impairments of spermatogenesis

Groups by Histological and Cytological Analysis	No. of Biopsy Specimens Tested	No. (%) of Biopsy Specimens Expressing the Gene				
		<i>GCL</i>	<i>CDY1</i> minor	<i>DAZ</i>	<i>RBM1</i>	β -Actin
Normal spermatogenesis	19	17 (90)*	19 (100)	19 (100)	19 (100)	19 (100)
Hypospermatogenesis	23	15 (65)	16 (70)	20 (87)	20 (87)	23 (100)
Spermatocyte maturation arrest	10	6 (60)	2 (20)	9 (90)	9 (90)	10 (100)
Sertoli cell only	15	6 (40)*	0 (0)	0 (0)	0 (0)	15 (100)

* Significant difference between the 2 groups ($P = .003$, Fisher's exact test).

Table 2. Individual results of biopsy specimens studied for meiotic impairments*

Serial No.	Groups by Histological and Cytological Analysis	Rate of Bivalent Formation		Expression of HGCL	Biopsy Specimens With Spermatozoa, %
		X-Y	18-18		
1	Normal spermatogenesis	79	95	–	100
2		89	97	+	100
3		90	98	+	100
4		86	98	+	100
5		83	98	+	100
6	Hypospermatogenesis	94	100	–	100
7		60	88	–	33
8		89	99	–	100
9		93	100	–	100
10		84	98	+	100
11		80	99	+	100
12		78	86	+	50
13		68	96	+	100
14		89	99	+	100
15		71	77	+	100
16	82	98	+	100	
17	62	94	+	100	
18	Spermatocyte maturation arrest	46	85	+	100
19		28	71	+	0
20		15	61	+	0
21		19	40	+	0
22		35	64	–	0

* The rate of bivalent formation (in percent) of chromosomes X-Y and 18, human germ cell less (HGCL) expression, and percentage of biopsy specimens with spermatozoa found in the specific testis are displayed. Plus sign indicates HGCL present; minus sign, HGCL absent.

ing and non-HGCL-expressing ones ($P = .039$). Although motility is difficult to assess when performing ICSI, testicular specimens with weakly motile, nonmotile, or a low percentage of motile spermatozoa were clearly detected among 6 (75%) of 8 men in the hypospermatogenesis group with the absence of HGCL expression. These findings were different from men in the same group who expressed HGCL, among whom only 4 (27%) of 15 had motility impairments. Yet, there were 2 biopsy specimens from the normal group with no detectable HGCL who had normal sperm motility and morphology.

HGCL Expression and Chromosome Bivalents Formation in Spermatocytes

As a transcription repressor expressed mainly at the pachytene spermatocytes in mouse, GCL might regulate meiosis. We therefore tested for a possible correlation between HGCL expression and meiosis bivalent formation in the testicular biopsy specimens. Table 2 depicts individual results of the bivalent formation rate of homologous chromosomes X-Y and 18, the HGCL expression findings, and the presence of testicular spermatozoa in at least 1 of the 3 biopsy samples of the testis. There was a significantly higher rate of bivalent formation of homologous chromosomes (both X-Y and 18) wherever spermatozoa were found in at least one location of the testis (Pearson correlation, $P < .001$). The rate of chromosome bivalent formation was not affected by the presence or

absence of HGCL expression (Table 2). The rate of bivalent X-Y chromosomes (mean \pm SE) was 66.8 ± 6.43 and 75 ± 9.52 in the groups with and without GCL expression, respectively, and the rate of 18 chromosome bivalent was 87.1 ± 4.26 and 91 ± 5.71 , respectively.

Discussion

The HGCL gene was variably expressed in biopsy specimens with different impairments of spermatogenesis. The extent of its expression was in parallel to the severity of the spermatogenetic impairment, as indicated by the most advanced stage of the germ line. Defective sperm motility was significantly associated with the absence of HGCL expression.

A significant association between HGCL expression in the testis and the presence of germ cells or mature spermatid or spermatozoa was demonstrated. The detection of HGCL expression in 40% of the biopsy specimens with Sertoli cell only was intriguing, because its homologous mouse gene mGCL was detected in germ cells but not in somatic (eg, Sertoli) cells of the testis (Kimura et al, 1999; Leatherman et al, 2000). It is possible that HGCL expression might reflect abnormal events in the somatic cells of the testis. Studies using immunohistochemical analysis with anti-HGCL antibodies may clarify these findings. Unfortunately, polyclonal anti-mGCL antibodies

did not work on paraffin-embedded human testicular biopsy specimens, so the possibility that HGCL expression might serve as a marker for testicular abnormalities could not be tested.

Patients with germinal failure might have minute foci of spermatogenesis sparse throughout the entire testis (Silber et al, 1997). Side by side, different histological and minced tissue findings such as Sertoli-cell-only and complete spermatogenesis might be detected in such patients. In view of the nonhomogeneous nature of the testis, we compared the expression of *HGCL* to the expression of *DAZ*, *RBMI*, and *CDY1* minor genes within the same biopsy specimen. This was done in view of the fact that expression of *DAZ* and *RBMI* confirms the presence of spermatogenic cells (Menke et al, 1997; Elliot et al, 1998; Lee et al, 1998; Bar-Shira Maymon et al, 2001), whereas *CDY1* minor, expressed in spermatids, reflects the presence of haploid germ cells (Kleiman et al, 2001; Lahn et al, 2002). The expression of *HGCL* correlates with that of *DAZ*, *RBMI*, and *CDY1* minor. Nevertheless, exceptions were detected, suggesting that *HGCL* expression is more frequently impaired compared with the other markers tested. Further mutation analysis of the *HGCL* gene might help to clarify the source of the impairment, either mutations on the gene or impaired regulation of gene expression.

It was estimated that approximately 2000 different genes are involved in a variety of testicular functions, including testicular development, germ cell differentiation, meiosis, and spermiogenesis (Bhasin et al, 1998), suggesting that the genetic basis of male infertility might be highly heterogeneous. We classified our biopsy specimens based on the overall histological and cytological testicular findings: these groups could include subgroups with different unknown mutations that lead to similar impairment, a feature that might complicate the interpretation of our results.

Most biopsy specimens with spermatozoa were found to express *HGCL*, and defective sperm motility was observed very frequently whenever no *HGCL* was expressed, suggesting that *HGCL* might be involved in the regulation of differentiation during spermiogenesis. Larger numbers of patients and a quantitative analysis of motility would be needed to test this assumption. Many factors might influence the testicular sperm motility detected. However, the recently published findings in *mgcl-1*-null male mice model support our observation (Kimura et al, 2003). Structural abnormalities, decrease of sperm motility, and reduction of path velocity of motile sperm were observed in *mgcl-1*-null male mice.

Recent studies performed on transfected H1299 cells have reported an intrinsic and additive ability of mGCL to repress transcription (Nili et al, 2001b). Other proteins (eg, LAP2 β) might compensate for the absence of GCL

in certain cells or under specific circumstances and might explain the lack of critical impairments of spermatogenesis found whenever *HGCL* was absent.

Lessons from other species (mice, flies, etc) are usually helpful in understanding human infertility in general and for the function of *HGCL* in particular. Studies on *mGCL* suggested that GCL might play a role in cell cycle, particularly at meiosis (de la Luna et al, 1999; Kimura et al, 1999; Leatherman et al, 2000). No particular difference, however, was observed in the rate of X-Y and 18 chromosome bivalents in spermatocytes between biopsy specimens that expressed or failed to express *HGCL*, suggesting that *HGCL* was not involved or, at least, was not essential in the normal process of chromosome bivalent formation during meiosis. Recently, it was published that the first abnormality observed during spermatogenesis in *mgcl-1*-null male mice was abnormal nuclear envelope structure in spermatocytes, affecting the appropriate nuclear-laminar organization. This in turn is essential for normal sperm morphogenesis and chromatin remodeling during spermiogenesis (Kimura et al, 2003).

In conclusion, *HGCL* by itself seems to play a minor role in spermatogenesis, probably during spermiogenesis. *HGCL* expression was affected mainly when spermatogenesis was dramatically impaired. It did not appear to be involved in men with a prominent meiotic impairment. *HGCL* does not seem to be essential for spermatozoa production: it might merely affect the overall normal spermatogenesis process and spermiogenesis in a specific way, by influencing the activity of genes that are directly involved in these processes.

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