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Genetic Variations of *gpx-4* and Male Infertility in Humans[1]

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ABSTRACT

Phospholipid hydroperoxide glutathione peroxidase (PHGPx), the product of *gpx-4,* is the major selenoprotein in sperm and is considered essential for fertilization because of its multiple roles in spermatogenesis, such as hydroperoxide detoxification, formation of the mitochondrial capsule, and chromatin condensation. Genomic DNA sequences of 3.148 kilobases covering the whole *gpx-4* and its flanking regions were amplified from 63 men using the polymerase chain reaction and were analyzed for polymorphisms by direct sequencing. A total of 23 variant sites were detected; 2 were present only in control men (proven fathers; n = 21) and 10 were common to fertile controls and infertile patients (n = 42). A further 11 variant sites were seen in five of the infertile men only. Four of the *gpx-4* variants were considered irrelevant to GPx-4-related fertility problems because they occurred homozygously in controls. The majority of the remaining variant sites are also of questionable relevance because they are located in introns or, as third base exchanges, do not affect the protein sequence. However, one of the exon variations leads to an Ala93-Thr exchange that reduces activity in a porcine GPx-4 homologue. Two detected promoter variations were shown by reporter gene constructs to affect transcription in somatic cell lines. These results indicate that *gpx-4* polymorphism cannot generally account for the correlation of PHGPx content of sperm and fertility-related parameters, but further examination of this gene as a potential cause of infertility in particular cases is warranted.

gamete biology, gpx-4, male sexual function, PHGPx, selenium, sperm, spermatogenesis

INTRODUCTION

Phospholipid hydroperoxide glutathione peroxidase (PHGPx; GPx-4, E.C. 1.11.1.12) is one of the four selenocysteine-containing mammalian glutathione peroxidases (GPx) that, together with cysteine-containing homologues, belongs to a protein family spread over all living domains [1]. The catalytic cycle of these enzymes comprises oxidation of the redox-active selenium moiety and its stepwise reduction by thiol compounds, typically glutathione (GSH), as summarized in Equations 1–3:

$$\text{GPx-Se}^- + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{GPx-SeOH} + \text{H}_2\text{O} \quad (1)$$

$$\text{GPx-SeOH} + \text{GSH} \rightarrow \text{GPx-Se-SG} + \text{H}_2\text{O} \quad (2)$$

$$\text{GPx-Se-SG} + \text{GSH} \rightarrow \text{GPx-Se}^- + \text{GSSG} + \text{H}^+ \quad (3)$$

PHGPx is peculiar in reducing a wide variety of hydroperoxides ranging from H_2O_2 to hydroperoxo groups of complex lipids in biological membranes [2]. Because of this specificity, the enzyme was thought to act as an antioxidant device specifically designed to protect biomembranes against oxidative stress [3]. However, high levels and gonadotropin-dependent expression of PHGPx in testis [4, 5] pointed to a more distinct biological role.

In testicular tissue, *gpx-4* (the gene encoding PHGPx) is expressed in three different forms. By use of alternative start codons, the enzyme is targeted to the cytosol, mitochondria, or the nucleus [6, 7]. In situ hybridization [8, 9] revealed that *gpx-4* is abundantly expressed in late spermatogenic cells. Accordingly, most of the PHGPx activity of testis can be attributed to spermatids. PHGPx activity, however, is hardly detectable in mature spermatozoa. A switch in the redox status during late sperm maturation can explain this unexpected finding. By unknown processes, GSH disappears completely and cellular protein thiols become gradually oxidized [10, 11]. PHGPx, thus deprived of its usual reductant GSH, uses protein thiols as alternative substrates. It thereby becomes cross-linked with itself and other proteins via selenadisulfide bridges. Up to this point, the process is homologous to the usual enzymatic function according to Equations 1 and 2. However, by replacing GSH with a protein SH group, a dead-end intermediate (GPx-Se-S-Protein) is formed that is not easily reduced by a second GSH in a reaction similar to Equation (3) [12]. In consequence, an insoluble, keratin-like enzymatically inactive material is generated from which active PHGPx can only be rescued by long-term exposure to low-molecular-weight thiols [13, 14]. The oxidative PHGPx inactivation is obviously a crucial step in sperm maturation. The keratin-like material thus formed is the capsule that embeds the mitochondrial helix in the midpiece of spermatozoa [14]. By a similar mechanism, the nuclear form of PHGPx is believed to contribute to chromatin condensation [7, 15].

These novel insights explain the essential nature of the trace element selenium for male fertility that has been known for decades from research with livestock [16] and experimental rodents [17]. When PHGPx, which is the major selenoprotein of spermatogenic cells and spermatozoa, can no longer be adequately formed because of severe selenium deficiency, fertilization capacity is lost because of impaired mobility, morphological midpiece alterations, and detachment of heads and tails. Chimeric mice that had at least 50% *gpx-4* (+/−) cells displayed patchy testicular at-

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rophy, with a few spermatozoa showing alterations resembling those known from animals with selenium deficiency [18]. Clinical relevance of the experimental data is underscored by recent trials. PHGPx content of human sperm, measured as rescued PHGPx activity, was positively correlated with sperm count, motility, and structural integrity [19], and the minute amounts of active PHGPx left over in mature spermatozoa were correlated with fertility-related parameters [20].

Unlike livestock in selenium-deficient areas, humans in a civilized country do not usually suffer from a selenium deficiency pronounced enough to affect testicular PHGPx biosynthesis. The often dramatic deficiency in sperm PHGPx content observed in infertile men [19] must therefore have other causes, such as genetic defects of *gpx-4* itself or of the complex system of selenoprotein synthesis or alterations of the unclear mechanisms leading to timely and tissue-specific overexpression of *gpx-4*. We screened patients who consulted an andrology unit for fertility problems, and proven fathers were used as controls for polymorphisms of the *gpx-4* gene. The sequence of this gene was highly variable in both healthy controls and infertile men. However, a few quite unusual variations were found in infertile men only.

MATERIALS AND METHODS

Patients and Control Men

The study was conducted on 63 Caucasian men; 42 were infertile patients and 21 were fertile controls (proven fathers). The etiology of infertility appeared plausible for 22 patients. Infertility in the remaining 20 patients was classified as idiopathic. Semen analysis was performed as described previously [19] for all but three of the patients. Diagnosis of oligospermia, cryptospermia, or azoospermia corresponded to sperm counts of $<20 \times 10^6$ sperm/ml, $<5 \times 10^5$ sperm/ml, or no detectable sperm, respectively, in three independent semen analyses. Asthenozoospermia was diagnosed if sperm motility was reduced to <50%. Diagnostic data and potentially relevant findings, as available from clinical routine diagnosis, are compiled in Table 1. Histological confirmation of a spermatogenic disorder was only obtained for five patients. Fifteen of the infertile patients were selected from the study on rescued PHGPx activity in sperm [19]. The Ethical Committee of the Medical Faculty of the University of Padova approved the study, and the participants gave their informed consent.

DNA Amplification by Polymerase Chain Reaction

A total of 3.148 kilobases of the *gpx-4* gene, including 335 nucleotides of the promoter region [21], were scanned by direct sequencing of six partially overlapping amplicons obtained by polymerase chain reaction (PCR) with DNA purified from whole blood according to the method of Miller et al. [22]. For amplification and sequencing, the following primers were used (size of amplified fragment in parentheses): 1) 5′-ttatacaagcggctcagcaa-3′ and 5′-ggacttgttgttggcgactg-3′ (977 base pairs [bp]) and sequencing primers 5′-cccctcgacgagtctta-3′ and 5′-catgctcgcttgtgtctagg-3′; 2) 5′-agttggagaaaccaaaccc-3′ and 5′-tagtcaggactgcgcgact-3′ (998 bp); 3) 5′-ctcaggctccagtgaccttgg-3′ and 5′-atgtccttggcggaaaactc-3′ (1034 bp) and sequencing primer 5′-actgcaaggcaaaggaag-3′; 4) 5′-agatccacgaatgtcccaag-3′ and 5′- ctccacagccctctatgtcc-3′ (1097 bp) and sequencing primers 5′-gcacattttggggttgga-3′ and 5′-tgtgagagtcccagcacct-3′; 5) 5′-cgggctacaacgtcaaattc-3′ and 5′-gggtgcacgctggattttc-3′ (1000 bp); 5′-acttcaccaaggtaaggggg-3′ and 5′-agatctcactgcacccca-3′ (704 bp) and sequencing primer 5′-ctctccacagttcctcatc-3’.

Amplification reactions were performed on a Perkin Elmer-Cetus (Überlingen, Germany) thermocycler. The four deoxynucleotide triphosphates (0.2 mM each), primers (0.8 μM each), genomic DNA (75 ng), *Pfu* DNA polymerase (0.6 U; Promega, Madison, WI), the manufacturer's buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 2.5 μg BSA), and dimethyl sulfoxide (8%) were contained in a total volume of 12.5 μl. After covering the reaction mixture with one drop of mineral oil, thermal cycling was performed with an initial denaturation at 95°C for 2 min, followed by 38 cycles of denaturation at 95°C for 30 sec, primer annealing at 62–65°C for 30 sec, and primer extension at 74°C for 3 min. The final extension was carried out at 74°C for 5 min. Quality of amplification was examined on a 1.5% agarose gel before sequencing.

DNA Sequencing and Analysis

The amplification products were transferred onto a 96-well microtiter plate, treated for 15 min at 37°C with exonuclease I (5 U) and alkaline phosphatase (1 U), denatured at 80°C for 15 min, and processed for sequencing using Big Dye dideoxy-terminator chemistry (Perkin Elmer) on an ABI 3700 sequencer (Perkin Elmer). Data were visualized using Chromas (available at http://bmr.cribi.unipd.it). Only high-quality sequences were considered. Each chromatogram was visually inspected for possible heterozygous variants. The obtained sequences were compared to the reference sequence (accession AC004151) using the ''dialing 2'' and ''boxshade'' sequence alignment programs (http://www.gene-regulation.com/pub/programs.html).

Sequences of newly obtained amplicons were confirmed at least once for each variable site by sequencing in both directions. Heterozygous insertion/deletion variants were cloned from the pertinent PCR products in a TA vector (Clontech, Palo Alto, CA). Thereafter, both strands of at least four different clones were resequenced.

Preparation of PHGPx Variants

The pCYTEXp1-based vector for expression of porcine *gpx-4* variants was used to study the putative relevance of PHGPx amino acid substitutions, as described previously [23]. The vector containing the complete cDNA encoding porcine PHGPx was mutated by means of the Quickchange site-directed mutagenesis kit (Stratagene, San Diego, CA) according to the manufacturer's instructions. The TGA codon for selenocysteine was mutated to TGT to allow expression of the cysteine homologue (PHGPx U46C) in *Escherichia coli*. The double mutant PHGPx U46C/A93T was obtained accordingly. Correctness of the intended mutations in the *gpx-4* variants was confirmed by sequencing both DNA strands. The mutant genes were expressed in *E. coli* TG2. The products were isolated by bromosulfophthalein-Sepharose chromatography and gel filtration as described previously [23] and concentrated in an ultrafiltration cell with a 10-kDa cutoff membrane (Pall Gelmann Laboratory, Dreieich, Germany). More than 90% homogeneity was verified by SDS-PAGE (Phast System; Amersham Biosciences, Buckinghamshire, U.K.). For activity measurements according to the method of Ursini et al. [2], the proteins were passed through a Sephadex G25 column to adjust buffer conditions (0.1 M Tris-HCl, 5 mM EDTA, pH 7.6).

Promoter Efficiency Studies

Luciferase reporter plasmids were constructed by cloning 348 bp (from nucleotides −303 to +45) of the supposed promoter *gpx-4* region [21] in the *Bgl*II/*Kpn*I site of the pGL3 Basic plasmid (Promega GmbH, Mannheim, Germany) by conventional techniques. The reference promoter sequence and the mutated promoter sequences of participants 7, 39, 56, or 26 were obtained by PCR with the primers 5′-ggtaccagttggagaaaccaaacccc-3′ and 5′-agatctctcgtccagccgaccaat-3′, and template DNA from participant 37 (reference promoter) or participants 7 or 26 (mutated promoters), respectively. After band purification and ligation with the appropriately digested pGL3Basic vector, the correctness of the resulting constructs (designated pGLB Ref, pGLB 7/39/56, pGLB 26, respectively) was verified by sequencing in both directions.

LNCaP, T47D, and MCF7 cells were grown in RPMI 1640, Dulbecco modified Eagle medium (DMEM), or DMEM plus 2 mM glutamine and nonessential amino acids, with 10% fetal calf serum and 50 nM sodium selenite and antibiotics at 37°C and 5% CO_2 in a humidified incubator. For transient transfection, cells were seeded in 3.5-cm six-well plates and grown to 70% confluence. Transfection was carried out using ExGen 500 (MBI-Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. Cells were cotransfected with 1 μg of the above reporter plasmid and 1 μg of a β-galactosidase expression vector to standardize for transfection efficiency, incubated for 24 h, harvested, washed twice in PBS, and lysed by the addition of 200 μl of lysis buffer (Luciferase assay system; Promega GmbH, Mannheim, Germany). Luciferase activity was quantified on a 10-μl sample by a Packard tri-carb liquid scintillation analyzer. The β-galactosidase activity was measured spectrophotometrically at 420 nm on a 20-μl sample previously incubated for 30 min as previously described [24].

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TABLE 1. Clinical data for participants in the study

Participant no.	Diagnosis	Classification according to semen analysis	Other findings*
1	Idiopathic	Oligoasthenospermia	
2	Idiopathic	Asthenospermia	
3	Varicocele	Asthenospermia	
4	Varicocele	Asthenospermia	
5	Varicocele	Asthenospermia	
6	Varicocele	Asthenospermia	
7	Idiopathic	Asthenospermia	
8	Idiopathic	Asthenospermia	
9	Idiopathic	Asthenospermia	
10	Idiopathic	Oligoasthenospermia	
11	Idopathic	Asthenospermia	
12	Varicocele	Oligoasthenospermia	3
13	Fertile		
14	Varicocele	Asthenospermia	9
15	Obstructive infertility	Normospermia	3
16	Inguinal ernia	Oligoasthenospermia	3
17	Idiopathic fertile	Asthenospermia	9
18			
19	Idiopathic	Oligospermia	1, 2, 9
20	Idiopathic	Aspermia	1, 3
21	Y deletion	Chryptospermia	
22	Idiopathic	Oligospermia	1, 3, 4
23	Cryptorchidism	Aspermia	
24	Cryptorchidism	Oligospermia	3
25	Fertile		
26	Idiopathic	Cryptospermia	3
27	Cryptorchidism	Aspermia	
28	Idiopathic	Asthenospermia	3
29	Y deletion		3
30	Cryptorchidism	Oligospermia	3
31	Cryptorchidism	Crypstospermia	3, 5
32–39	Fertile		
40	Idiopathic	Oligospermia	9
41	Y deletion	Cryptospermia	9
42	Idiopathic	Cryptospermia	3
43	Post-mumps orchitis	Aspermia	3
44	Idiopathic	Aspermia	3, 6
45, 46	Fertile		
47	Male turner syndrome		3
48	Varicocele	Oligospermia	3
49	Idiopathic	Oligospermia	3
50	Varicocele	Normospermia	9
51	Idiopathic	Oligospermia	
52	Idiopathic	Asthenospermia	3
53	Varicocele	Cryptospermia	3
54, 55	Fertile		
56	Idiopathic	Aspermia	3, 6, 7
57	Erectile dysfunction		8, 9
58–63	Fertile		

* 1, Hypospermatogensis; 2, disturbed sperm maturation; 3, testicular volume ≤15 ml; 4, arrest of sperm maturation; 5, female kind of obesity associated with high FSH and low testosterone levels (15.4 mIU/ml and 2.4 ng/ml, respectively); 6, Sertoli cells only; 7, increased testicular consistency; 8, gynecomastia; 9, testicular volume >15 ml.

RESULTS

Identification of Novel gpx-4 *Alleles*

Only 17 men did not show any variation compared with the reference sequence. In the remaining 46 men, 23 different variant sites were identified (Table 2). Of these, 2 were found in the control group only, 10 were common to controls and the infertile men, and 11 were found in the infertile men only. Among these novel alleles, insertion/deletion variants were less common than substitution variants (13% of the observed variations). Among the substitutions, transitions were more common than transversions (70% vs. 30%). Four substitution variants mapped the promoter region (positions −221, −197, −180, and −100), and nine mapped the exons, i.e., two for the 5′ untranslated region (UTR) of the mRNA, four for the coding region, and three for the 3′ UTR of the gene. Of the four variants in the coding region, one is a second base substitution leading to the replacement of the serine in position 2 in the mitochondrial leader sequence by asparagine (G → A, nucleotide +82), another one (G → A, nucleotide +1725) results in a change from alanine to threonine in the mature protein, and the remaining two are silent third-position substitutions. A total of 10 variants were found in introns at sites that were not expected to affect splicing [25].

Cases of Homozygosity

Homozygosity was detected for five novel alleles (Table 3). These comprised the most frequent alleles: the silent G

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TABLE 2. Genetic variants identified in *gpx-4* for fertile and infertile men.

		Allele frequency‡				
			Infertile patients			
Position*	Variant†	Fertile controls (n = 21)	Known etiology (n = 22)	Unknown etiology (n = 20)	Total (n = 63)	Participant no.
−221	g → c	0	0	0.050	0.016	26
−197	a → t	0.048	0	0.100	0.048	7, 39, 56
−180	c → t	0.048	0	0.100	0.048	7, 39, 56
−100	c → t	0	0	0.050	0.016	26
+6	C → T	0	0	0.050	0.016	26
+17	G → A	0	0	0.050	0.016	26
+82	G → A	0.048	0	0	0.016	39
+113	G → A	0.285	0.273	0.300	0.286	1, 10, 21, 23, 24, 26, 29, 30, 34, 35, 40, 42, 43, 44, 45, 59, 61, 63
+341	c → t	0	0.045	0	0.016	57
+400	t → c	0	0	0.050	0.016	26
+473	g → c	0	0	0.100	0.032	7, 56
+909	C → T	0.143	0.090	0.150	0.126	2, 19, 33, 35, 36, 48, 50, 52
+1345	t → c	0	0	0.050	0.016	26
+1658	g insertion	0.286	0.182	0.250	0.238	1, 21, 24, 26, 29, 34, 36, 40, 42, 43, 44, 45, 59, 61, 63
+1725	G → A	0	0.045	0	0.016	31
+1881–1886	ggggtg deletion	0.143	0.227	0.150	0.174	2, 4, 8, 16, 18, 27, 33, 47, 51, 53, 54
+1943	g insertion	0.048	0	0.050	0.032	17, 36
+2142	g → t	0.095	0.136	0.100	0.111	1, 13, 21, 30, 40, 43, 45
+2148	g → c	0	0	0.050	0.016	26
+2512	g → c	0.428	0.363	0.350	0.380	1, 2, 10, 13, 21, 23, 24, 25, 26 29, 30, 31, 33, 34, 39, 40, 42, 43, 44, 45, 50, 59, 61, 63
+2650	T → C	0.619	0.50	0.60	0.571	1, 2, 3, 4, 7, 8, 10, 13, 14, 18, 19, 21, 24, 25, 30, 32, 33, 34, 35, 36, 40, 42, 43, 44, 45, 47, 48, 50, 51, 52, 53, 54, 56, 59, 61, 63
+2663	C → T	0.048	0	0	0.016	38
+2767	G → A	0	0.045	0	0.016	57

* The position refers to the reference sequence (accession AC004151), where position +1 is the uppermost transcription start site so far identified [31].
† Variant sites are indicated as substitution or insertion/deletion with respect to the reference sequence. Variant sites within the introns or the promoter region are shown in lower case; those within the exons in capital letters.
‡ Allele frequency has been calculated for each group (fertile controls, infertile subjects with known or unknown etiology) and for the whole study group (total).

→ A transition in exon 1, the g insertion in intron III, two single-base polymorphisms in introns IV and VI, respectively, and the T → C transition at nucleotide +2650 belonging to the 3′ UTR. This transition is located upstream of the SECIS element, which is essential for selenocysteine insertion, and is thus of questionable functional relevance (Table 3 and Figs. 1 and 2). Four of the five cases of homozygosity were detected in proven fathers and in infertile patients. Accordingly, these *gpx-4* variants cannot account for infertility. The impact on fertility of the fifth homozygous variation, the transversion in intron IV (position +2142), is uncertain because although it was detected in an infertile patient it was also found in a heterozygous state in controls.

Heterozygous gpx-4 *Variants Occurring in Controls and Infertile Patients*

A total of 12 variants sites were detected in both controls and infertile patients (Fig. 1 and Table 2). Two of these variants were detected only in proven fathers, thus representing variants not likely associated with infertility. The first is the G → A transition of nucleotide +82 resulting in a Ser → Asn substitution in the mitochondrial import sequence. This N-terminal extension, which is cleaved off in the mature protein, is rich in basic amino acids, a condition required for mitochondrial import [26]. Therefore, the observed variation should affect neither the enzymatic activity of the mature PHGPx nor PHGPx mitochondrial import. The second variation detected in proven fathers only was the C → T transition at nucleotide +2663, which, like the

TABLE 3. Homozygous sequence variations detected in *gpx-4* for fertile and infertile men.

Position	Variant	Frequency of homozygosity*	Participant no.†
+113	G → A	0.22	21, 34, 42, 61
+1658	g insertion	0.33	21, 26, 34, 42, 61
+2142	g → t	0.14	21
+2512	g → c	0.208	1, 21, 42, 45, 61
+2650	C → T	0.22	1, 2, 21, 33, 34, 36, 42, 61

* Fraction of those participants displaying the particular allele.
† The number identifies the specific participant carrying the homozygous variation (Table 2). The reported variations were detected homozygously in both fertile and infertile men, except for the variant at +2142, which was carried as a homozygous substitution by only one patient, whose infertility was ascribed to chromosome Y deletion.

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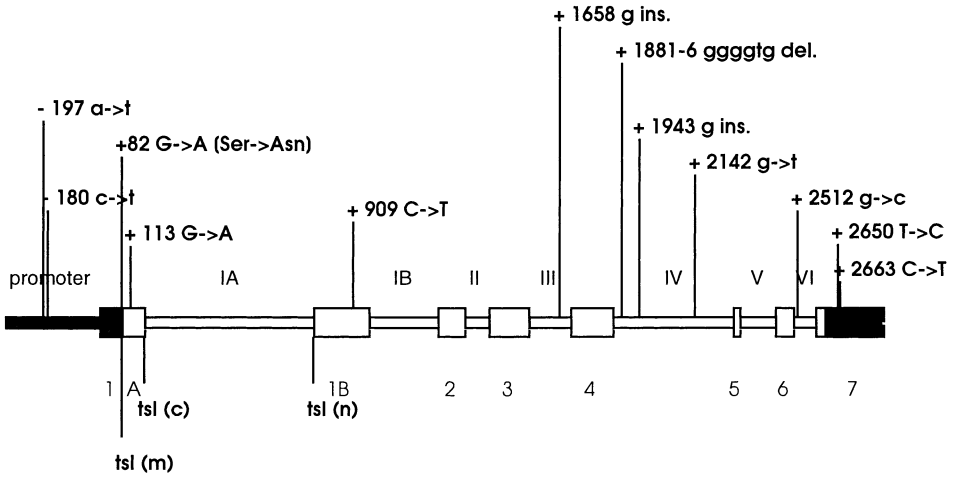

FIG. 1. Summary of transversally distributed *gpx-4* variants. The *gpx-4* variations found in controls or common to fertile and infertile men are demonstrated in context of the gene structure. The exons are represented as big boxes with Arabic numbers. Introns are indicated by Roman numbers. The filled boxes represent promoter, the 5′ UTR, or the 3′ UTR, respectively. The two alternative exons yielding the mitochondrial/cytosolic or nuclear protein are indicated as 1A and 1B respectively. The translational starts for the mitochondrial, cytosolic, and nuclear proteins are indicated as tsl (m), (c), and (n) and occur at positions +78, +159, and +769 respectively. TGA for SECIS insertion at positions +1437/+1439 in exon 3. SECIS at positions +2670/+2711 in exon 7. For allele frequencies, see Table 2.

T → C change at nucleotide +2650 is located in the 3′ UTR outside of sequence stretches that are known to be functionally important. The other 10 variants are 1) the 4 frequent variants that were also seen in homozygous state in proven fathers, 2) the variant at nucleotide +2142 found as a homozygous variation in one infertile patient, and 3) 5 more variants that are not suggestive of any effect on *gpx-4* expression or PHGPx function because they are distributed among controls and infertile patients. However, because none of these varaints were found in the homozygous state, an impact on fertility cannot be excluded. Two of such variants are seen in the promoter region and thus may affect *gpx-4* expression in general or its tissue specificity, and the quite frequent C → T transition at nucleotide +909 in exon 1B is silent. Two of the heterozygous polymorphisms are located in the large intron IV, the 6-bp deletion of nucleotides 1881–1886 and a g insertion at nucleotide +1943. The functional relevance of this intron variability remains elusive.

Gpx-4 *Variants Seen Only in Infertile Patients*

A total of 11 variant sites were detected in the *gpx-4* gene of infertile patients only (Fig. 2). They comprise two single-base pair polymorphisms of the promoter region, two variations in the 5′ UTR of the mRNA, five seemingly irrelevant variations in introns IA, II, and IV, respectively, the G → A transition at nucleotide +1725 resulting in Ala93-Thr exchange at position 93 of the amino acid sequence, and an A → G transition at nucleotide +2767 in the 3′ UTR located downstream of the SECIS element, which comprises the nucleotides from +2670 to +2711.

These 11 additional variants were found clustered in the genes of five patients. None of those differed from the reference gene by a single variation only. The variations uniquely seen in infertile patients were combined with either transversally distributed or unique variations (Fig. 3). One of multivariant genes was detected twice in unrelated men (nos. 7 and 56) from different areas in Italy. The g → c transversion in intron IA is combined with three transversally distributed single nucleotide polymorphisms (SNPs). Both patients were suffering from idiopathic infertility attributed to asthenozoospermia or azoospermia, respectively. Patient 57, who presented to the hospital for erectile dysfunction, had two unique SNPs. Patient 31, diagnosed as cryptospermic, is the only one so far detected with a sequence alteration in the processed PHGPx. The most extraordinary case is patient 26, with idiopathic cryptospermia, who displayed 10 *gpx-4* variations, 7 of which are unique. Two of these variations are located in the promoter region, the one at nucleotide 100 mapping to an Sp 1 site [21], two other variations are in the 5′ UTR of the mRNA, and three more are in introns IA, II, and IV.

Potential Functional Relevance of Ala93-Thr Exchange in PHGPx

Although the Ala93-Thr exchange in patient 31 is located far from the active site, its functional relevance was tested by means of an established expression system that

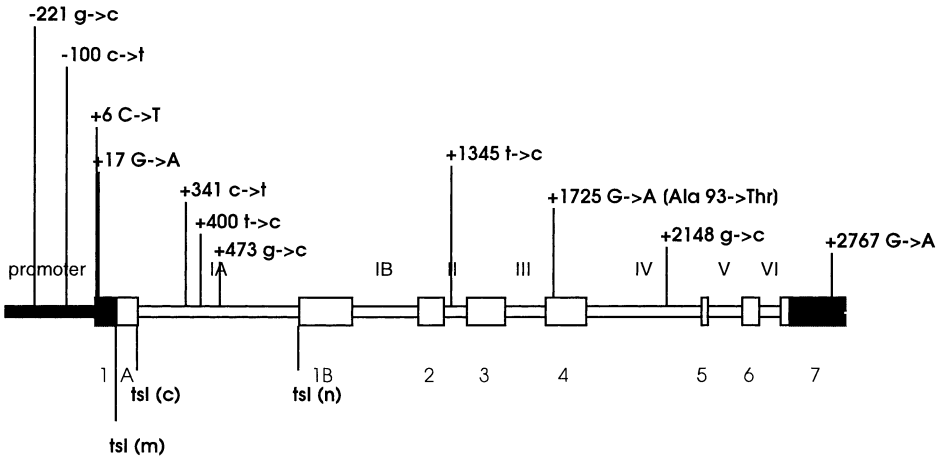

FIG. 2. Compilation of *gpx-4* variants found only in the infertile patients. The variants were found in five different patients and were exclusively heterozygous substitutions. Except for the variation at position +473, which was recorded in two different patients, the others were recorded only once in each but were clustered in three different patients.

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Subjects # 7 and # 56:

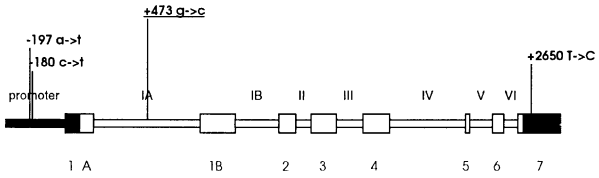
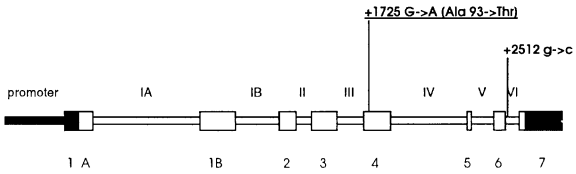


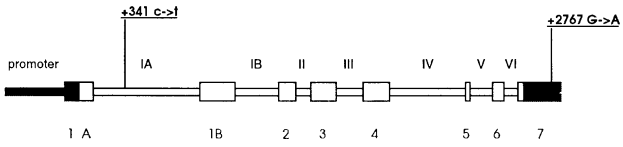
FIG. 3. Variant *gpx-4* maps of the five infertile patients. The variant sites of each infertile patient carrying polymorphisms not shared with the control group are underlined. Some patients also carry variations shared with controls (not underlined). Semen analysis was performed on the ejaculates of four men but was not available for patient 57, who visited the hospital for erectile dysfunction. For clinical data, see Table 1. Patient 7 also participated in the study on rescued sperm PHGPx specific activity, which was 26% of control levels.

Subject # 26:

Subject # 31:



Subject # 57:



had been successfully used for the characterization of the catalytic center of porcine PHGPx [23]. Expression of selenoproteins in mammalian cells with reasonable yields is still a technically unsolved problem, and mammalian selenoprotein genes cannot be expressed in prokarya because of differences in the translation system [27]. The selenocysteine codon must therefore be mutated into a cysteine codon to enable an overexpression in *E. coli*. The resulting cysteine homologue and its further mutations can be isolated by a fast and convenient chromatographic procedure and tested for specific activity [23]. In agreement with previous findings [23], the PHGPx U46C mutant thus prepared showed a low specific activity of 0.12 U/mg. The double mutant PHGPx U46C/A93T displayed an even lower specific activity of only 0.02 U/mg, which suggests that the Ala-Thr exchange may be relevant to activity in the homologous human selenoprotein.

Potential Relevance of Promoter Variations

The two variant genes containing SNPs in the promoter region (Fig. 3) were checked for functional relevance by means of conventional reporter gene constructs in transiently transfected human cell lines T47D and MCF7, which are

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TABLE 4. Activity* of the reference and mutated PHGPx promoter constructs in different cell lines.

Construct	Cell type		
	LNCaP	MCF7	T47D
pGLB Ref	59.8 ± 40.5	35.2 ± 83.2	234.5 ± 104.0
pGLB 7/39/56	175.0 ± 68.4†	729.7 ± 170.0‡	545.0 ± 238.4§
pGLB 26	1138.6 ± 643.5†	3766.9 ± 548.9‡	2730.5 ± 987.3‡

* Numbers represent the ratio between luciferase (c.p.m) and β-galactosidase activity (optical density × 10^3) (mean ± SD of six independent experiments).
† $P < 0.009$ versus the reference promoter.
‡ $P < 0.002$.
§ $P < 0.023$.

derived from breast cancer cells, and LNCaP, which is derived from prostate cancer cells. The reporter genes comprised the promoter and flanking sequences from nucleotide −303 to nucleotide +45 linked to an in-frame firefly luciferase gene, which implies that in case of the reporter gene construct derived from patient 26, the two variations of the first exon also were included. The corresponding stretch of a ''normal'' promoter (from participant 37) was used for the construction of a reference vector (pGLB Ref). As compiled in Table 4, the promoter, as mutated in participants 7, 39, and 56 (pGLB 7/39/56), was at least twice as active in all three cell lines when compared with the reference promoter. The heavily mutated 5′ area of patient 26 (pGLB 26) exceeded the activity of the reference promoter by a factor of 10–100.

DISCUSSION

The human *gpx-4* gene was highly variable in both normal control individuals and patients suffering from fertility problems. SNPs, insertions, and deletions were observed. The incidence of SNPs detected in the *gpx-4* exons (1 SNP per 87.5 bases) is actually 20 times that predicted for chromosome 19, where *gpx-4* is located [28, 29]. The overall incidence of polymorphisms did not differ significantly between diagnostic groups, although it tended to be higher in patients with idiopathic infertility (Table 2). The *gpx-4* genes from 17 of 20 patients with idiopathic infertility were identical to the reference sequence or contained transversally distributed variations only. Thus, *gpx-4* polymorphisms cannot be a frequent cause of fertility problems. This outcome of our pilot study is not surprising despite the strong correlation between PHGPx content of sperm and infertility [19, 20]. As discussed in detail elsewhere [1], PHGPx biosynthesis in testis may be negatively affected by many factors, such as severe selenium deficiency [17], intoxication by heavy metals [30], insufficient hormonal stimulation [4], or any other disruption of the poorly understood signaling cascade specifically triggering the burst of expression in spermatids. Moreover, in many cases a low PHGPx content of sperm may be the result rather than the cause of impaired spermatogenesis, because the PHGPx content of sperm was also depressed in infertile patients with known etiologies, such as postmumps orchitis or testicular trauma [19].

Nevertheless, the unusual clustering of multiple and partly unique variations seen in five infertile men (Fig. 3), three of which were diagnosed with idiopathic infertility, speaks in favor of a possible relationship between gene-based impairment of PHGPx expression or function and infertility. However, final proof is lacking and will remain difficult to provide for several reasons. Homozygous cases of defective PHGPx genes cannot be expected because *gpx-4* disruption in mice is lethal when homozygous (M. Brielmeier, personal communication). In consequence, PHGPx mutations leading to loss of function should be extremely rare. PHGPx activity in somatic cells cannot be used for preliminary screening because it is not correlated with testicular expression [20]. The mechanisms and the genetic elements determining the specific *gpx-4* expression in spermatids are unknown, and suitable models for molecular analysis are not yet available. Of the three distinct roles of PHGPx in spermatogenesis, as a peroxidase [2], a structural protein [13], and a factor for chromatin condensation [7, 15], only the first one is reasonably understood and can be measured in vitro. Accordingly, our attempts to functionally characterize the most intriguing *gpx-4* variants can at best stimulate further investigations.

The only PHGPx sequence variation (Ala93-Thr) detected (in cryptospermic patient 31) could indeed explain infertility if the porcine model mutant PHGPx U46C/A93T mimics the corresponding human selenoprotein. Based on the preliminary experiments with transgenic mice [18], the decreased activity of the mutant might impair spermatogenesis even in a heterozygous state. The mutation, which is located opposite to the enzyme's active site, might affect enzyme polymerization, which is required for sperm maturation [13].

The results of the reporter gene experiments, which were designed to investigate the relevance of the two detected promoter SNPs, remain puzzling. They reveal that the variations do affect *gpx-4* expression, although they do so in an unexpected direction. In three lines of somatic cells, the mutated promoter detected in participants 7, 39, and 56 was about twice as active as the reference promoter, and the mutant promoter of patient 26 was >10 times as active. These findings do not support a reduced expression in spermatogenic cells. However, because expression varies substantially between the cell lines (Table 3), such promoters may behave differently in spermatogenic cells in situ, where *gpx-4* expression rises from barley detectable to very high levels [8, 9]. Unfortunately, there is no cell system available that would reliably mimic the differentiation into spermatids, where *gpx-4* expression is triggered physiologically.

The reporter gene experiments are further limited in not considering additional downstream variations. The promoter mutations seen in patients 7 and 56 were also found in a healthy man but in another genetic context and are therefore not necessarily equivalent. The downstream variations in the *gpx-4* of patient 26, with a total of 10 variant sites, certainly cannot be ignored. It is hard to imagine that such a highly variable gene would still comply with the complex regulatory demands of spermatogenesis.

In the majority of infertile patients, the *gpx-4* gene cor-

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responds to the reference gene, and a number of frequently occurring *gpx-4* polymorphisms were ruled out as potential causes of fertility problems. However, a few cases of multivariated *gpx-4* genes were detected as heterozygous alleles in infertile patients only. One of the mutations alters the sequence of PHGPx, probably leading to reduced activity. Two patients had two mutations each in the promoter region combined with two or eight additional downstream variations, respectively. Being functionally relevant in principle, such mutations might disturb the regulated *gpx-4* expression during spermatogenesis. Further screening of *gpx-4* genes of infertile patients for mutations might unravel the intriguing role of PHGPx in spermatogenesis.

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