

·Original Article·

Testicular expression of *survivin* and human telomerase reverse transcriptase (*hTERT*) associated with spermatogenic function in infertile patients

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Abstract

Aim: To characterize the coexpression of *survivin*, an inhibitor of apoptosis (IAF), and human telomerase reverse transcriptase (*hTERT*) in human testes with varying spermatogenic function. **Methods:** Transcript levels of *survivin* mRNA and *hTERT* mRNA were determined in normal testes ($n = 11$) and testes with defective spermatogenesis ($n = 28$) using real-time reverse-transcription polymerase chain reaction (RT-PCR). The histological work-up was performed according to a modified Johnsen score. **Results:** Expressions of both *survivin* and *hTERT* were highest at median levels of 96.8 and 709 in normal spermatogenesis and dropped to 53.3 and 534 in testes with postmeiotic spermatogenic arrest ($n = 10$). In severe spermatogenic failure ($n = 18$), *survivin* expression was lacking in most specimens ($n = 16$), whereas at least low levels of testicular *hTERT* expression were largely detectable with a normalized expression of 73 in premeiotic spermatogenic arrest ($n = 7$) and 45 in patients with Sertoli cell-only syndrome (SCOS) ($n = 3$). Both *survivin* and *hTERT* expressions increased with a progressing Johnsen score (P for trend = 0.001). **Conclusion:** Although both *survivin* and *hTERT* are correlated with spermatogenic function, they show different expression patterns in testes of infertile patients. These findings substantiate results from studies in the rodent testis suggesting a predominant expression of *survivin* in meiotically dividing germ cells. (*Asian J Androl* 2006 Jan; 8: 95–100)

Keywords: *survivin*; human telomerase reverse transcriptase; apoptosis; azoospermia; male infertility; spermatogenesis

1 Introduction

Normal production of germ cells is dependent on a precise balance of both proliferation and apoptotic cell death. The molecular mechanisms underlying the regu-

lation of germ cell homeostasis in the testis are only partly understood. Genes involved in growth and proliferation as well as pro- and anti-apoptotic factors seem to play important roles in this context. Impaired proliferative capacity and dysregulated apoptosis can contribute to human spermatogenic disorders [1]. Genetic alterations, such as impaired expression of anti-apoptotic genes, are thought to be involved in idiopathic fertility defects [2–4].

Both *survivin* and *telomerase* are candidate regulatory genes that might affect proliferation and apoptosis in tissues with extensive cellular proliferation, such as

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the seminiferous epithelium. Telomerase activity has been linked to high proliferative capacity and correlates with the expression of the catalytic subunit of the telomerase enzyme, human telomerase reverse transcriptase (*hTERT*). In gonadal tissues, telomerase activity is restricted to specific germ cells, that is, spermatogonia, pachytene spermatocytes and round spermatids [5]. Evidence of a crucial role for telomerase in the maintenance of male fertility has come from animal models [6]. In humans, decreased telomerase activity [7–10] and impaired *hTERT* expression [9] have been linked to spermatogenic disorders. Thus, dysregulated expression of telomerase may be involved in spermatogenic failure.

Among the mammalian apoptosis regulators, the inhibitor of apoptosis (IAP) protein family is just beginning to be elucidated for its role in spermatogenic function. *Survivin* is an IAP protein regulating apoptosis at cell division. *Survivin* has been studied extensively in cancer cells, but has only recently received serious attention in male germ cells. In the rodent testis, *survivin* expression has been localized to germ cells, especially mature spermatocytes [11, 12]. Relatively high levels of *survivin* expression have been demonstrated in normal human spermatogenesis and its downregulation associated with spermatogenic failure [13]. Taken together, these studies suggest that *survivin* is a candidate regulatory gene in male germ cell production.

Recent evidences of telomerase [14] and *survivin* [12] involvement in the meiotic division of male rodent germ cells led us consider whether comparable expression patterns of these genes could be found at the mRNA level in human testes with varying spermatogenic function. The aim of this investigation was to determine the quantitative coexpression of *survivin* mRNA and *hTERT* mRNA in testicular biopsies from men with normal spermatogenesis and specific spermatogenic disorders.

2 Materials and methods

2.1 Patients

All investigated tissue specimens were collected from patients presenting with azoospermia-related infertility at the Department of Urology, Charité–Universitätsmedizin Berlin, Campus B. Franklin, Berlin, Germany, and the Department of Andrology, University of Hamburg, Hamburg, Germany, between 1997 and 2002. The use of the specimens was approved by the Ethics Committee of the Free University of Berlin. All patients gave their informed con-

sent prior to the biopsy. Two semen analyses per patient were carried out according to the World Health Organization (WHO) guidelines [15]. Morning baseline serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were determined prior to biopsy. Non-obstructive azoospermia was clinically diagnosed in the majority of patients investigated in this study.

2.2 Processing of the biopsy material

For testicular biopsy, a 3-mm³ tissue sample was obtained through a small incision in the tunica albuginea and processed as previously described [13]. Briefly, the biopsy material was divided into several fractions. The largest part was reserved for a possible intracytoplasmic sperm injection and immediately placed in Sperm-freeze solution (Medicult, Hamburg, Germany). A small fraction of the specimen was intended for molecular investigation of gene expression by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). It was shock-frozen immediately after removal then stored at –80 °C. Another small part of the specimen was placed in Stieve's solution and served as material for the histopathological investigation, which was performed according to a modified Johnsen score [16]. This score describes the preservation of spermatogenesis on a scale from 1 (no germ cells or Sertoli cells) to 10 (intact spermatogenesis).

2.3 RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from the biopsy material using RNeasy (WAK Chemie Medical, Bad Homburg, Germany) according to the manufacturer's instructions. The concentration and quality of RNA (28S/18S ratio) were determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany).

Quantitative real-time fluorescence RT-PCR was performed using the LightCycler instrument (Roche Molecular Systems, Alameda, CA, USA) according to the manufacturer's instructions. In a one-step RT-PCR reaction, 250 ng total RNA were subjected to cDNA synthesis and subsequently amplified during 40 PCR cycles (0.5 s at 95 °C; 10 s at 60 °C; and 10 s at 72 °C). The mRNA encoding *hTERT* and the housekeeping porphobilinogen deaminase (*PBGD*) gene were processed in the same way. The final concentration of MgCl₂ was 6 mmol/L for all PCR reactions. The respective primers for *survivin* (GenBank accession number AF077350) were sense 5' -

AAA GAG CCA AGA CAAAA TTG C-3' and antisense 5' -GAG AGA GAA GCA GCC ACT GTT AC-3' generating a 338-bp product. The hybridization probes used are specific for an internal segment of the amplified *survivin* fragment (FL probe: TGC TCT TGT TTT GTC TTG AAA GTG GC—FL and LC Red640 probe: CCA GAG GTG CTT CTG CCT GTG C—PH). The primers, probes and standards for *hTERT* and *PBGD* analyses were supplied with the LightCycler Telo TAGGG *hTERT* Quantification kit (Roche Diagnostics, Mannheim, Germany) and the *h-PBGD* Housekeeping Gene Set (Roche Diagnostics, Mannheim, Germany), respectively.

To carry out a positive control and to establish an external standard curve, all measurements included the determination of standards for *survivin*, *hTERT* and *PBGD*. The *survivin* cRNA standard was generated using a cloned *survivin* cDNA fragment as described [17].

The LightCycler 3.3 (Roche Diagnostics, Mannheim, Germany) was used to analyze PCR kinetics and to calculate quantitative data. In each sample, copy numbers of *survivin* mRNA and *hTERT* mRNA were normalized by copy numbers of *PBGD* mRNA. Samples lacking housekeeping gene expression were excluded from analysis.

2.4 Statistical analysis

To compare expression data of all histological groups, the Kruskal–Wallis test for non-parametric analysis of variance (ANOVA) and the Jonckheere–Terpstra test for trend were performed using SPSS 10.5 (SPSS Inc., Chicago, IL, USA). Significant differences between two groups were assessed with the Mann–Whitney *U*-test or Fisher's exact test, as appropriate. Regression coefficients were computed to detail the correlation between gene expression and histopathological findings (Johnsen score).

3 Results

The histopathological work-up provided evidence of full spermatogenesis (Johnsen score 8–10, obstructive azoospermia) in 11 patients. Twenty testicular biopsies showed partial tubular atrophy with maturation arrest. Postmeiotic maturation arrest (MA) corresponding to a Johnsen score of 5–7 was seen in 10 of these cases. Histopathological findings in the remaining 10 samples were consistent with premeiotic spermatogenic arrest corresponding to a Johnsen score of 3–4. Histopathology demonstrated germ cell aplasia (Sertoli cell-only syn-

drome [SCOS], Johnsen score 2) in eight patients. Serum FSH, LH and testosterone levels were within the normal reference range in patients with normal spermatogenesis. Patients with spermatogenic failure showed high variability of serum FSH with a mean \pm SD of (17.3 \pm 10.8) mIU/mL (range 4.0–34.2 mIU/mL). Neither the LH nor the testosterone levels differed significantly from those in the subgroup with normal spermatogenesis.

PBGD expression levels did not differ significantly between the histological groups (Kruskal–Wallis ANOVA: $P = 0.1$). The median (range) *PBGD* expression was 10 486 copies (3 495–87 530) in tissue specimens with SCOS, 30 765 copies (7 609–81 770) in those with premeiotic MA, 56 120 copies (3 82 –107 300) in those with postmeiotic MA, and 47 270 copies (14 160–201 800) in those with normal spermatogenesis. Comparisons of individual groups disclosed significant differences between SCOS and normal spermatogenesis ($P = 0.016$). This means that the criteria for an ideal internal standard was only partially met by *PBGD*. SCOS specimens may thus show a slight distortion towards higher normalized gene expression values.

Survivin mRNA expression was measured in all of the biopsy specimens. Using serial dilutions of the *survivin* cRNA standard, the detection limit of the assay was determined to be 10^3 copies. *Survivin* mRNA expression was detectable in all specimens with normal spermatogenesis ($n = 11$) and in the majority (9 of 10) of those with postmeiotic MA (Johnsen score 5–7). In contrast, only 2 of the 10 specimens with premeiotic MA (Johnsen score 3–4) evidenced *survivin* mRNA expression. None of the samples from SCOS patients showed detectable *survivin* mRNA expression in the RT-PCR despite the presence of *PBGD* expression. Except in two specimens from patients with premeiotic spermatogenic failure, *survivin* mRNA expression was thus restricted to samples containing meiotically dividing or postmeiotic germ cells. Table 1 depicts the descriptive statistics of quantitative *survivin* mRNA expression in human testicular tissues with varying spermatogenic function. *Survivin* levels were highest in specimens with normal spermatogenesis and were decreased in those with a postmeiotic MA. Significant differences in testicular *survivin* levels were observed between the histological groups of testicular biopsies (Kruskal–Wallis ANOVA, $P < 0.001$) with positive correlations between normalized *survivin* values and Johnsen scores (Figure 1, P for trend < 0.001). Comparison of individual groups disclosed differences be-

tween premeiotic arrest and postmeiotic arrest (Fisher's exact test, $P = 0.013$) as well as between postmeiotic MA and normal spermatogenesis (Mann-Whitney U -test, $P = 0.031$). Figure 1 illustrates the distribution of quantitative *survivin* expression by histology groups.

Quantitative *hTERT* mRNA expression was analyzed in 31 specimens (SCOS, $n = 3$; premeiotic MA, $n = 7$;

postmeiotic MA, $n = 10$; normal spermatogenesis, $n = 11$). The detection limit for *hTERT* analysis is 10^2 copies. *hTERT* mRNA expression was detected in all but one specimen with SCOS. Expression levels were highest in specimens with normal spermatogenesis and lowest in those with SCOS (Table 1). The Kruskal-Wallis ANOVA showed significant differences in testicular *hTERT* values between

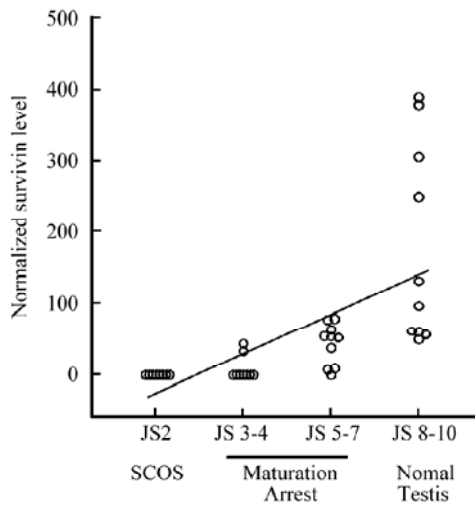


Figure 1. Normalized *survivin* expression levels in normal testes and spermatogenic disorders. All cases with Sertoli cell-only syndrome (SCOS) and most cases with premeiotic maturation arrest (MA) tested negative for *survivin* expression. A significant trend towards higher *survivin* levels was observed with an increasing Johnsen score(JS) (P for trend < 0.001). Linear regression revealed that testicular *survivin* mRNA levels were positively associated with the JS ($r = 0.63$).

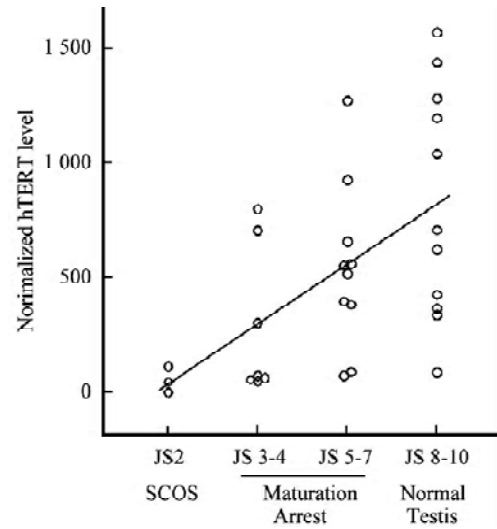


Figure 2. Normalized human telomerase reverse transcriptase (*hTERT*) expression levels in normal testes and spermatogenesis disorders. A significant trend towards higher *hTERT* levels was observed with an increasing Johnsen score (P for trend < 0.001). Linear regression revealed that testicular *hTERT* levels were positively associated with the Johnsen score (JS) ($r = 0.56$).

Table 1. Quantitative *survivin* mRNA and *hTERT* mRNA expression in human testicular tissues with varying spermatogenic function. *hTERT*, human telomerase reverse transcriptase; MA, maturation arrest; SCOS, Sertoli cell-only syndrome.

Histology	Johnsen score	<i>n</i>	Mean	Median	25 th ; 75 th percentiles	Range
<i>Survivin</i>						
SCOS	2	8	0.0	0.0	0.0 ; 0.0	0.0–0.0
MA						
Premeiotic	3–4	10	7.8	0.0	0.0 ; 8.3	0.0–44.4
Postmeiotic	5–7	10	43.6	53.3	8.4 ; 67.1	0.0–79.6
Normal	8–10	11	166.4	96.8	57.1 ; 305.2	50.3–388.9
<i>hTERT</i>						
SCOS	2	3	52.3	45.0	0.0 ; 112.0	0.0–112.0
MA						
Premeiotic	3–4	7	291.4	73.0	55.0 ; 705.0	46.0–798.0
Postmeiotic	5–7	10	542.0	534.0	309.0 ; 724.0	72.0–1271.0
Normal	8–10	11	824.2	709.0	363.0 ; 1282.0	86.0–1568.0

the histological groups ($P = 0.011$). Normalized *hTERT* levels were positively correlated with the Johnsen score (Figure 2, P for trend = 0.001). However, differences were not statistically significant when individual histological groups were compared (Mann–Whitney U -test, $P > 0.05$). Testicular *hTERT* levels were lower in patients with spermatogenesis disorders than those in subjects with full spermatogenesis ($P = 0.015$). Owing to the relatively low housekeeping gene expression in SCOS samples, *hTERT* mRNA expression in these specimens may be lower than calculated. This should not interfere with the data on differences between *hTERT* and *survivin* expression patterns, but it may mask differences in *hTERT* levels between SCOS and other histological subgroups.

4 Discussion

The present study describes coexpression of the *survivin* and the telomerase subunit *hTERT* in human testicular tissue. Largely consistent with our findings, results of previous studies demonstrated decreased testicular telomerase activity in patients with spermatogenic failure [7, 9, 10]. However, telomerase activity appears to be present throughout the seminiferous epithelial cycle up to the round spermatid stage, at least in rat testis [5]. Thus, marked differences in testicular *hTERT* expression between specimens with MA and those with normal spermatogenesis seem counter-intuitive at first. However, they may be partly attributable to the fact that specific telomerase-positive cells like spermatogonia express *hTERT* at lower levels in testes with spermatogenic failure than in those with normal spermatogenesis. Moreover, given that the *hTERT* promoter is activated by estrogens [17], changes in the paracrine environment in patients with spermatogenic failure may also contribute to decreased testicular telomerase activity. These potential regulative mechanisms warrant further investigation.

Corroborating previous preliminary data [13], we confirmed that the level of *survivin* mRNA expression is correlated with the severity of spermatogenic failure in infertile patients. In contrast to telomerase expression, however, that of *survivin* seems to be restricted to specimens containing meiotically dividing or haploid germ cells. These findings are in line with results in rodents demonstrating a preponderance of *survivin* protein and mRNA expression in meiotically dividing spermatocytes [11, 12].

Taken together, these studies raise the question of *survivin* involvement in the meiotic progression of germ cells. On the other hand, our results cannot exclude *survivin* expression by other cell types, for example, spermatogonia or even Sertoli cells, in the normal testis. Consistent with the role of *survivin* in the mitotic cell cycle, low levels of expression are found in spermatogonia in rats [12]. Thus, it remains to be elucidated which germ cell types actually express *survivin* in humans.

Interestingly, we found coexpression of *survivin* and telomerase only in specimens characterized by the presence of haploid germ cells. In contrast, *survivin* expression was lacking despite the expression of *hTERT* in specimens showing severe spermatogenic failure. Telomerase expression thus seems to relate to the cellularity or proliferative capacity of the seminiferous epithelium. On the other hand, recent evidence suggests additional roles of telomerase in male mammalian meiosis [6, 14]. Germ cells may require functional telomerase for both proliferation and meiotic division. Our study disclosed a relatively high variability of transcript levels within the histological subgroups, especially in the subgroup with normal spermatogenesis. This may indicate an influence of factors unrelated to the spermatogenic function on the expression levels of these genes. However, all patients with normal testicular histopathology had obstructive azoospermia. It is not yet clear whether even higher and less variable expression levels may occur in fertile men. It must also be considered that the observed differences between testicular *survivin* and telomerase expression could be due to artifacts. RT-PCR analysis of *survivin* mRNA may just lack the sensitivity to detect low expression levels of this IAP in premeiotic germ cells, such as spermatogonia, or even Sertoli cells. Indeed, low levels of *survivin* may be expressed in Leydig cells in the rodent testis [12]. Moreover, the described expression pattern of *survivin* mRNA remains to be confirmed at the protein level by other detection strategies such as immunohistochemistry.

Low or no expression of anti-apoptotic genes such as *survivin* might play a role in the pathogenesis of spermatogenic disorders, as increased germ cell apoptosis has been observed in conjunction with impaired human spermatogenesis [1]. It remains to be elucidated whether altered *survivin* and/or telomerase expression are causative factors in the development of spermatogenic disorders. Overexpression of *survivin* in colon cancer cells has recently been shown to enhance telomerase

activity by the upregulation of *hTERT* expression [18]. This opens up new perspectives on the question of germ cells having intimately linked *survivin* expression and telomerase activity to inhibit apoptosis and to prolong their cellular lifespan. Our findings indicate that testicular coexpression of *survivin* and *hTERT* is associated with normal spermatogenesis or postmeiotic maturation. Testes lacking haploid germ cells show *hTERT*, but not *survivin* mRNA expression, which supports the finding of predominant *survivin* expression during meiosis [11, 12].

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