LDOC1 Gene Expression in Men With Klinefelter Syndrome

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Klinefelter syndrome (KS) results from an extra chromosome X, which is due to the failure of normal chromosomal segregation during meiosis. Patients with KS have gynecomastia, small testes, and azoospermia. Apoptosis is a mechanism responsible for the normal regulation of spermatogenesis. *LDOC1* gene is a known regulator of nuclear factor mediated pathway to apoptosis through inhibition of nuclear factor kappa B (NF-kappaB). Furthermore, the transcription factor myeloid zinc finger gene 1 (MZF-1) has been shown to interact with *LDOC1* and to enhance *LDOC1* activity favoring apoptosis. We investigated the expression of *LDOC1* gene mRNA, by quantitative reverse transcription polymerase chain reaction (qRT-PCR), in peripheral blood leukocytes of 13 patients with KS compared to 13 healthy men chosen as controls. *LDOC1* expression was higher in 9 of the 13 KS patient compared to normal controls. These finding led us to hypothesize that *LDOC1* gene upregulation may play a role in the spermatogenesis derangement observed in patients with KS. J. Clin. Lab. Anal. **30**: 408–410, 2016. © 2016 Wiley Periodicals, Inc.

Key words: apoptosis; azoospermia; Klinefelter syndrome; *LDOC1* gene; qRT-PCR

INTRODUCTION

Klinefelter syndrome (KS), initially described in 1942 (1), is due to the presence of an extra X chromosome (2). The syndrome occurring in about 1 in 600 male newborns is the most common heterochromosome abnormality (3). Approximately 80% of the patients with KS carry the numerical chromosome aberration 47,XXY; the remaining 20% have higher grade chromosome aneuploidies (48,XXXY) or mosaicisms (4). From the clinical standpoint, patients with KS have small testes, azoospermia, gynecomastia, low normal levels of testosterone, and high levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (5). KS may be associated with an increased risk of systemic diseases (6).

Apoptosis is a mechanism responsible for the normal regulation of germ-cell death during maturation and differentiation of normal human germ cells. Hence, apoptosis is a prerequisite for continuous spermatogenesis by selectively removing dysfunctional or damaged germ cells, and by limiting germ cell number (7, 8). On these premises, apoptosis may contribute to the excessive germ-cell demise in men with 47,XXY with ensuing azoospermia.

The leucine zipper, downregulated in cancer 1 (*LDOC1*) gene maps on chromosome X (q27) and consists of one exon (OMIM 300402). It encodes for a nuclear protein, the 146-amino acid *LDOC1* protein, with a calculated molecular mass of approximately 17 kDa, which contains a leucine zipper-like motif in its N-terminal region and a proline-rich region that shares marked similarity to an SH3-binding domain (9). Northern blot analysis detected ubiquitous expression of *LDOC1* gene in normal tissues, with high expression in brain and thyroid and low

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expression in placenta, liver, and leukocytes (10). The wide expression of *LDOC1* mRNA in normal tissues and the absence of *LDOC1* in most pancreatic and gastric cancer cell lines indicate that downregulation of *LDOC1* gene may have an important role in the development and/or progression of some cancers (10). *LDOC1* is a known regulator of nuclear factor kappa B (NF-kappaB) that can affect the phorbol 12-myristate 13-acetate (PMA) or tumor necrosis factor α (TNF-α) mediated pathway to apoptosis through inhibition of NF-kappaB (10). Furthermore, myeloid zinc finger gene 1 (MZF-1), a transcription factor, has been shown to interact with *LDOC1* and to enhance *LDOC1* proapoptotic activity (11). In addition, the expression of Wiskott–Aldrich syndrome protein family, member 3 (WAVE3), induces the translocation of *LDOC1* from the nucleus to the cytoplasm, resulting in the inhibition of *LDOC1*-induced apoptosis, thus, it is possible to envisage that *LDOC1* function is negatively regulated by WAVE3 (12). On this basis, we develop interest in investigating the expression of *LDOC1* gene mRNA, by qRT-PCR, in peripheral blood leukocytes of 13 patients with KS. The results were compared with mRNA LDOC-1 gene expression of 13 healthy men chosen as controls.

MATERIAL AND METHOD

The study was conducted in 13 patients with KS with a mean age of 30.9 ± 11.4 years (range 21–60 years) and in 13 normal control men with mean age of 30.4 ± 11.1 years (range 21–59 years). The diagnosis of KS was made after 10 years because of the presence of small testis (volume <1 ml bilaterally), despite a normal puberty. Hormonal evaluation resulted in slight hypotestosteronemia and increased LH and FSH serum levels. Karyotype analysis was 47,XXY ever since the patients have been on testosterone replacement therapy with long-acting testosterone esters. Other than this, the patients are in a good health, does not smoke nor drinks alcoholic beverages. The KS patients and controls were recruited after personal informed consent. RNA was extracted from peripheral blood leukocytes using RNeasy Mini Handbook (Qiagen Sciences, Germantown, PA), following the manufacturer's protocol. RNA quantity and quality were checked by spectrophotometry. To avoid any genomic DNA contamination during qRT-PCR, a brief incubation of the samples at 42°C with a specific Wipeout buffer (QuantiTect Reverse Transcription Kit, Qiagen Sciences) was carried out. Retro-transcription of 600 ng of total RNA from each samples was then performed in a final volume of 50 μl and generated cDNA was used as a template for real-time quantitative PCR analysis using gene expression products. For each sample, real time PCR reactions were carried out in duplicate, using 2.5 μl of cDNA and QuantiTect Probe PCR Master Mix Kit (Qiagen Sciences) in a total volume of 50 μl. Target gene *LDOC1* and reference gene *GAPDH* assays were obtained from Applied Biosystems (Carlsbad, CA). The thermal cycling conditions consisted of one cycle for 2 min at 50°C, one cycle of 15 min at 95°C and 40 cycles for 15 sec at 94°C followed by 1 min at 60°C. Real-time analysis was performed on an ABI PRISM 5700 Sequencer Detector (Applied Biosystems). The amplified transcripts were quantified using the comparative ΔΔCt method CT method (13); and relative quantification analysis data were obtained using the comparative ΔΔCt method included in the software version 1.3 supplied by the Applied Biosystems with *LDOC1*gene expression level normalized to *GAPDH* expression gene level and Target Mean Cp definition used for indicating the mean normalized cycle threshold.

TABLE 1. LDOC1 Gene Expression in KS and Normal Controls

Sample name	T. M. Cp (LDOC gene)	R. M. Cp (GAPDH gene)	Ratio normalized
N1	33.13	25.13	1.000
KS1	33.82	24.78	1.671
N2	37.80	30.23	1.000
KS2	40.87	33.75	1.372
N3	26.36	33.80	1.000
S3	27.20	33.51	0.458
N4	28.09	20.96	1.000
KS4	28.33	20.92	0.824
N5	30.05	24.52	1.000
KS5	30.25	22.87	1.189
N6	27.73	37.47	1.000
KS6	20.48	21.71	1.945
N7	26.96	32.65	1.000
KS7	29.59	21.70	4.594
N8	25.60	30.20	1.000
KS8	24.93	29.33	0.880
N9	29.08	22.86	1.000
KS9	29.61	20.83	5.810
N10	29.00	21.19	1.000
KS10	26.67	19.12	1.115
N11	31.65	30.98	1.000
KS11	31.05	30.72	1.251
N12	27.83	20.78	1.000
KS12	28.10	17.92	0.446
N13	29.25	31.32	1.000
KS13	29.11	31.31	1.112

T.M., target men gene; T.R., target reference gene; Cp, crossing points; N, normal subject; KS, Klinefelter syndrome subjects.

RESULTS

In this case–control study, *LDOC1* gene expression was increased in nine (69.23%) samples from KS subjects compared with age- and sex-matched control subjects (KS1, KS2, KS4, KS6, KS7, KS9, KS10, KS11, and KS13; Table 1. Two of them (KS7 and KS9 in

Table 1 have demonstrated the expression of at least four times compared to normal samples. Moreover, two of the nine samples of subjects KS (KS1 and KS6 in Table 1 showed an expression *LDOC1* greater than 1.5 compared to the corresponding normal sample.

DISCUSSION

Mechanisms of testicular degeneration in Klinefelter have been extensively studied and several mechanisms have been proposed. These include the following hypotheses: Leydig cell insufficiency, impaired somatic environment of the testes, a dysfunctional communication between somatic and germ cells, and apoptotic mechanism of Leydig and Sertoli cells (14). The expression data obtained in this case report study for LDOC1 underline the potential role that could have apoptosis in the onset of azoospermia as well as of inflammatory and immune-related diseases that are frequently encountered by patients with KS.

Overexpression of *LDOC1* cause externalization of the cell membrane phosphatidylserine, which is characteristic for early-phase apoptotic events (11). Mizutani et al. (12) have shown that ectopically expressed *LDOC1* is localized in the nucleus and induces apoptosis, accompanied by an increase in the tumor protein p53 (p53) protein level, but not in p53 transcription, suggesting that *LDOC1* inhibits the degradation of p53. *LDOC1* has been shown to inhibit the activation of NF-kappaB through ligand-induced stimulation by TNF-α or PMA in a dose-dependent manner and viability studies demonstrated that TNF-α or PMA-induced anti proliferative effects were significantly enhanced by stable transfection of cells with LDOC1 (10). These observations suggested that *LDOC1* was a novel regulator of NF-kappaB that can affect the PMA or TNF-α-mediated pathway to apoptosis through inhibition of NF-kappaB (10). Furthermore, Lynn et al. (15), in a study of expression array on to male young onset hypertension, have suggested that innate immune response and cell-proliferation regulation may play important downstream roles in development of hypertension and specifically that *LDOC1* gene plays a key role in the regulatory mechanisms related to apoptosis in hypertension. In addition, *LDOC1* gene expression was increased in Down syndrome subjects and in patients with Parkinson's disease (16, 17).

Our data suggest a potential role for *LDOC1* gene as a marker of the apoptotic mechanisms working in KS, however, the data obtained from our experiments need to be validated confirming the link between over expression of *LDOC1* and activation of the apoptotic pathways both in processes of KS.

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