Comparative expression of thioredoxin-1 in uterine leiomyomas and myometrium

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ABSTRACT: Uterine leiomyomas are benign tumors that develop from smooth muscle cells (SMCs). The reactive oxygen species (ROS) have been shown to be involved in the signaling pathways that stimulate proliferation of a variety of cell types. Thioredoxin-1 (TRX-1) is a redox-regulating protein, which is overexpressed in various tumors. In the present study, we investigated the expressions of TRX-1 and its related molecules in uterine leiomyomas and matched adjacent myometrium. Our results showed the expression of TRX-1 was increased in leiomyomas compared with the matched adjacent myometrium by quantitative RT–PCR and western blotting. FOXO3A expression was increased in leiomyomas compared with myometrium by western blotting. The mRNA levels of hypoxia-inducible factor-1α, cyclooxygenase-2 and cyclin D1 were increased in leiomyomas compared with the adjacent myometrium. The mRNA level of (thioredoxin-1-binding protein) TBP-2 in leiomyomas was not altered when compared with the matched adjacent myometrium. These results suggest that TRX-1 and some of its related molecules are associated with the pathogenesis of uterine leiomyomas. The identification of TRX-1 signaling pathways leading to cell proliferation points to another potential therapeutic target for treatment and/or prevention of uterine leiomyomas.

Key words: thioredoxin-1 (TRX-1) / hypoxia-inducible factor-1α (HIF-1α) / cyclooxygenase-2 (COX-2) / cyclin D1 / uterine leiomyoma

Introduction

Uterine leiomyomas are the most common pelvic benign tumors in women. Uterine leiomyomas can cause abnormal uterine bleeding, pressure symptoms, pelvic pain or reproductive dysfunction. They are extremely common, with prevalence rates of up to 70% in women of reproductive age (Tropeano et al., 2008). Uterine leiomyomas are characterized by an increase in smooth muscle cells (SMC) proliferation (Stewart et al., 1994; Walker and Stewart, 2005). A growing body of evidence indicates that leiomyomas growth is mediated by ovarian steroids (estrogen and progesterone) (Sadovsky et al., 1993; Rein et al., 1995). Growth factors have been reported to be associated with the growth of leiomyomas (Sawyn et al., 1995; Shimomura et al., 1998; Yu et al., 2008). More recent studies have shown that growth factors and cytokines can induce NADPH oxidase-dependent reactive oxygen species (ROS) production, which in turn has been shown to activate mitogen-activated protein kinases that regulate downstream cell proliferation or matrix production (Ushio-Fukai et al., 1998; Svegliati et al., 2005).

Thioredoxin-1 (TRX-1) is a ubiquitous protein, which operates together with NADPH and thioredoxin reductase as TRX system (Collet and Messens, 2010). TRX-1 has various biological activities, such as regulating the cellular redox balance, promoting cell growth, suppressing apoptosis and inflammation (Hashimoto et al., 1999; Masutani, et al., 2004; Yoshioka et al., 2006). TRX-1 is induced by various stimuli, such as H2O2, UV and reperfusion. TRX-1 expression is increased in various tumors, for example hepatocellular carcinoma, lung cancer, breast cancer and acute lymphocytic leukemia (Prowse and Montfort, 2001). However, the expression of TRX-1 in uterine leiomyomas has not been determined.

Therefore, the aim of this study is to compare the expressions of TRX-1 and TRX-1-related molecules in uterine leiomyomas and matched adjacent myometrium to further clarify the molecular mechanisms on proliferation of uterine leiomyomas.

Materials and Methods

Tissue collection and the ethics

Thirty women were recruited for this study, the patients ranged in age from 33 to 49 years old, none of the patients had received hormonal therapy...
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before surgery. All patients were hospitalized in the First Affiliated Hospital of Kunming Medical University, Kunming, China. Operation date was selected on the 7–10th day during menstrual cycle. All of the leiomyomas and normal myometrium biopsies were collected from patients who agreed to donate their removed tissues to the hospital. The patients are from Kunming. The leiomyomas and normal matched myometrium biopsies are from the same patient. The adenomyosis patients were excluded from the present study on the basis of tissue pathology. The leiomyomas tissues were from the center of leiomyoma and normal matched myometrium is 3 cm apart from leiomyoma. The types of surgery are total hysterectomy or subtotal hysterectomy. The ethics committee of the First Affiliated Hospital of Kunming Medical University approved this study and all procedures were performed in accordance with the Code of Ethics of the World Medical Association. Biopsies were immediately frozen in liquid nitrogen and stored at −80°C for later biochemical analysis.

Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from 0.1 g leiomyomas and normal myometrium tissues using a Trizol reagent kit (CWBIo Corporation, Beijing, China) and converted to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Waldorf Baden, Germany). The product was analyzed using a Primer 7300 Sequence Detection System (Applied Biosystems, Foster, CA, USA). The following primer pairs were selected for quantitative real-time polymerase chain reaction (PCR): human TRX-1 forward: 5′-AAG CCT TGG ACG CTG CAG-3′, reverse: 5′-CAT CCT GAC AGT CAT CCA CTA CTG-3′, probe: TGA TCA AGC CTT TCT TCT ATT CCC TCT C; human TBP-2 forward: 5′-GGA TCC CAG CAG TGC AAA C-3′, reverse: 5′-AAG CCG AAC TTT TAC TCA TAT TTG T-3′, probe: AGT ACC TGG GCT ATG AAG ACA CGC TT; human GAPDH forward: 5′-CAA GGC TGA CAG GGA CAA G-3′, reverse: 5′-GTT GAA GAC GCC AGT GGA CTG-3′; probe: ATC CCA TCA CCT TCT TCG AGT AGC G; human hypoxia-inducible transcription factor-1 (HIF-1α) forward: 5′-ACA GCA GCC AGA CCA TCG AG-3′, reverse: 5′-AAC TGG TCA GCT GTG GTA ATC CAC T-3′; human cyclooxygenase-2 (COX-2) forward: 5′-TGC ATT CTT TGC CCA GCA CTG-3′, reverse: 5′-AAA GGC GCA GGT TAC GTG GT-3′; human Cyclin D1 forward: 5′-GTG CGT CTG CAG AAT GGA ACG C-3′, reverse: 5′-ATC CAG GTG GCC ACG ACT T-3′. Reaction mixtures containing Premix Ex Taq™ (TaKaRa code: DRR039) and SYBR Green (TaKaRa code: DRR041; TaKaRa Biotechnology, Dalian, China) were prepared according to the manufacturer’s protocol. GAPDH was used as an internal standard for all samples.

Statistical analysis

Quantitative reverse transcriptase-polymerase chain reaction was used to analyze the mRNA level. Both leiomyomas and normal matched myometrium values were normalized to the internal control gene GAPDH, leiomyomas values represented the fold change relative to that of normal matched myometrium, which was converted to 1. The variance of the protein expression was normalized to β-actin. The variance of the protein expression was calculated as same mRNA expression.

All statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL, USA) software. The One-Way ANOVA analysis of variance with an LSD post hoc test adjustment was performed to compare variables. Comparisons of RT – PCR and western blotting between leiomyomas and normal myometrium were analyzed by multiple-samples comparison, respectively. And all statistical analysis data are expressed as the means ± SD. Differences were considered statistically significant at either *P < 0.05.

Results

TRX-1 expression was increased in leiomyomas

TRX-1 is related to proliferation in various cells. We firstly quantified the mRNA level of TRX-1 by using quantitative RT – PCR. The mRNA level of TRX-1 was significantly increased by 2.5-fold in leiomyomas examined (n = 30) compared with the matched adjacent myometrium (Fig. 1A).
Moreover, the protein level of TRX-1 was also significantly increased by 1.9-fold in leiomyomas (n = 30) when compared with the matched adjacent myometrium, which paralleled the mRNA level of TRX-1 (Fig. 1B and C).

**MDA concentration was increased in leiomyomas**

Many disorders are associated with overproduction of ROS. As a common by-product of lipid peroxidation, MDA is a well-accepted marker of oxidative stress. The levels of MDA in leiomyomas and matched adjacent myometrium tissues were examined by ELISA. The concentration of MDA was significantly increased by 2.4-fold in leiomyomas when compared with matched adjacent myometrium (n = 30) (Fig. 2).

**FOXO3A expression was increased in leiomyomas**

The forkhead-box (FOX) gene family of transcription factors, which includes > 100 members, includes FOXO3A that binds to the TRX-1 promoter to induce TRX-1 expression through the AMP-activated protein kinase pathway during oxidative stress responses (Li et al., 2009). Thus, we further examined FOXO3A expressions in leiomyomas and matched adjacent myometrium. We also found that the protein level of FOXO3A was significantly increased by 2.1-fold in leiomyomas examined when compared with the matched adjacent myometrium (n = 30) (Fig. 3).

**HIF-1α expression was increased in leiomyomas**

HIF-1α is overexpressed in various tumors, which is related to hypoxic response. Thus, we further examined hypoxia-inducible factor-1α (HIF-1α) expressions in leiomyomas and matched adjacent myometrium. We also found that the mRNA level of HIF-1α was significantly increased by 4.6-fold in leiomyomas, compared with the matched adjacent myometrium (n = 30) (Fig. 4A).

**COX-2 expression was increased in leiomyomas**

COX-2 is overexpressed in various tumors. Thus, we further examined COX-2 expressions in leiomyomas and matched adjacent myometrium tissues. We also found that the mRNA level of COX-2 was significantly increased...
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Cyclin D1 expression was increased in leiomyomas

Cyclin D1 is a necessary factor for the cell cycle and is overexpressed in various tumors. Thus, we further examined cyclin D1 expressions in leiomyomas and normal myometrium. We also found that the mRNA level of cyclin D1 was significantly increased by 2.8-fold in leiomyomas, compared with the matched adjacent myometrium (n = 30) (Fig. 4C).

Expression of TRX-1-binding protein in leiomyomas was same as adjacent myometrium

TRX-1-binding protein (TBP-2) has been reported to be a negative regulator of TRX-1 and have growth-suppressive activity. We also examined the TBP-2 expression by real-time PCR. However, there was no difference in the TBP-2 mRNA levels between leiomyomas and the matched adjacent myometrium (n = 30). The change variance was 1.1-fold (Fig. 4D).

Discussion

The uterine leiomyomas are benign uterine tumors characterized by increased SMC proliferation. It has been reported that ovarian steroids (estrogen and progesterone) influence the development and growth of uterine leiomyomas. Growth factors, such as epidermal growth factor (EGF), insulin growth factor, have been reported to be associated with the growth of leiomyomas (Shimomura et al., 1998). TRX-1 has important intracellular regulating roles in the signal pathway of estrogen (Deroo et al., 2004), as well as in the signal pathway regulated by EGF (Welsh et al., 2002). TRX-1 increases the proliferation of human B-cell lines through a protein kinase C-dependent mechanism (Biguet et al., 1994). Although TRX-1 is involved in mitogenic growth factor signaling pathways in various cells, TRX-1 regulation of human smooth muscle cell (SMC) proliferation in leiomyoma is still unclear.

In the present study, leiomyomas and the matched adjacent myometrial tissues were examined for expression of TRX-1. GAPDH has been commonly used as a single normalization gene in qRT–PCR studies. It also has been reported that most commonly used ‘housekeeping’ genes (HKG) are shown to vary considerably across samples and tissues (Andersen et al., 2004). GAPDH was the worst scoring candidate for normalization when comparing eutopic (Eu) and ectopic (Ec) endometrium sample groups. However, if only analyzing Eu endometrium, GAPDH was one of the two top-ranking candidate genes (Vestergaard et al., 2011). Based on the current knowledge and the samples in the present study were uterine tissues, GAPDH could be considered as a valid normalization gene. The relative protein expression was normalized to β-actin as previous study (Luo et al., 2006; Varghese et al., 2013).

Our result showed that increased expression of TRX-1 in leiomyomas compared with adjacent myometrium (Fig. 1A–C). Thus, the increased expression of TRX-1 in leiomyomas may be related to the proliferation of leiomyomas. However, our present result is different from Sahlin’s et al. (2000), in which TRX-1 was not related to leiomyomas. The difference may be due to different menstrual cycle of samples. Sahlin’s study samples were from proliferative phase, post-menopausal and pregnant. Our study samples are from proliferative phase.

On the other hand, uterine leiomyomas are benign uterine tumors characterized by extracellular matrix remodeling and increased collagen deposition. TRX-1 has a role in regulating the matrix metalloproteinase/tissue inhibitors of metalloproteinase balance (Antonietta et al., 2001). Moreover, collagen biosynthesis is under the regulation of TRX-1-dependent redox control (Matsumoto et al., 2002). Thus, the increased expression of TRX-1 may also be related to extracellular matrix remodeling in leiomyomas. ROS, once regarded as purely cytotoxic, are now recognized as effect-ive second messenger molecules regulating protein modifications, gene expression, cell proliferation, migration and differentiation as well as tissue remodeling in a variety of cell types (Ushio-Fukai et al., 1998). It has been reported that ROS mediate mitogenic growth factor signaling pathways in human leiomyoma SMCs (LSMCs). ROS are necessary components of the signaling pathways for platelet-derived growth factor and EGF in LSMCs (Mesquita et al., 2010). At the same time, many disorders are associated with overproduction of ROS, such as fibrotic conditions, pulmonary, hepatic and pancreatic fibrosis (Lambeth, 2007). Thus, we detected the concentration of MDA which is a common by-product of
Lipid peroxidation and is a well-accepted marker of oxidative stress. As we expected, the MDA concentration was higher in uterine leiomyomas compared with matched adjacent myometrium (Fig. 2). This result suggests that ROS is involved in oxidative stress in uterine leiomyomas in the present study.

The overexpression of forkhead-box protein O3A (FOXO3A) reduces the abnormal accumulation of ROS, enhances cellular resistance to oxidative stress and increases antioxidant gene expression (Li et al., 2010). Moreover, FOXO3A binds to the TRX-1 promoter to induce TRX-1 expression through the AMP-activated protein kinase pathway during oxidative stress responses (Li et al., 2009). Thus, the increased expression of TRX-1 may be regulated by FOXO3A. As we expected, FOXO3A expression was increased in uterine leiomyomas (Fig. 3). These results suggest FOXO3A/TRX-1 pathway is involved in ROS-regulating growth of uterine leiomyomas.

Mayer et al. (2008) reported that proliferating uterine leiomyomas in premenopausal women exhibited extremely low pO2 values (Mayer et al., 2008). Oxidative stress usually occurs in hypoxic conditions (Chi et al., 2010; Prabhakar et al., 2010). HIF-1α is induced under hypoxic conditions and oxidative stress. It has been reported that HIF-1α is induced by oxidative stress in the preterm delivery placenta (Song et al., 2012). In the present study, HIF-1α mRNA level was found to be significantly higher in uterine leiomyomas when compared with normal adjacent myometrium (Fig. 4A). The increase in HIF-1α expression may be associated with leiomyomas oxidative stress in uterine leiomyomas. Importantly, a redox-inactive TRX (C32S/C35S), an inhibitor of TRX-1, markedly decreases HIF-1α protein level, has been found to decrease the level of the HIF-1α transcription factor in tumors (Kim et al., 2011). Thus, the increase in HIF-1α expression may be associated with TRX-1 overexpression in uterine leiomyomas. However, there is also an inconsistent report that HIF-1α was negative in leiomyomas as well as myometrium, collected from premenopausal women (Mayer et al., 2008). The different results may be due to the samples from different sources. In the present study, our tissue samples are in the proliferative phase.

COX-2 is up-regulated in a variety of malignancies and does favor the growth of malignant cells by stimulating proliferation and angiogenesis (Stasinopoulos et al., 2013). COX-2 is up-regulated by TRX-1 (Csiki et al., 2006). We further examined COX-2 expression in leiomyomas. Our result showed the mRNA level of COX-2 was significantly higher in uterine leiomyomas when compared with matched adjacent myometrium (Fig. 4B). Thus, the increase of COX2 expression may be involved in SMCs proliferation in leiomyomas. Besides angiogenesis, COX-2 is an...
inducible enzyme that is up-regulated by the inflammatory responses. HIF-1α can be recruited by tissue inflammation (Jung et al., 2003a; Scortegagna et al., 2008). The increase of COX2 expression may be associated with inflammation occurred in leiomyomas (Maybin et al., 2011; Wegienka, 2012).

Cyclin D1 is overexpressed in various cancers and promotes the cell proliferation (Gillett et al., 1994). G1 progression entering into S phase was controlled by cyclin D1. Importantly, TRX-1 is an important regulator of the cell cycle in the G1 phase via cyclin D1 transcription and the extracellular regulated protein kinases/AP-1 signaling pathways (Mochizuki et al., 2009). Thus, cyclin D1 expression was also detected in the present study. Our data showed that the level of cyclin D1 mRNA was increased in leiomyomas (Fig. 4C). This result is consistent with previous study, in which ER α and cyclin D1 expressions were all elevated in comparison with adjacent myometrium (Kovács et al., 2001). The increased expression of cyclin D1 may promote the proliferation of SMCs.

TBP-2, also known as vitamin-D3 up-regulated protein-1 (VDUP1) or thioredoxin-interacting protein, has a molecular weight of 50 kDa. Thus, TBP-2 is the endogenous inhibitor of TRX-1 (Nishiyama et al., 1999; Junn et al., 2000; Patwari et al., 2006). TBP-2 plays important roles in cellular processes, including inhibiting cell growth, differentiation of myeloid and macrophage lineages (Kaimul et al., 2007). TBP-2 expression is decreased in various cancers, thus TBP-2 is known as an inhibitor of tumors (Ikarashi et al., 2002; Nishinaka et al., 2004; Ahsan et al., 2006; Takahashi et al., 2007). More importantly, TBP-2-deficient fibroblast cells proliferate more rapidly (Jeon et al., 2005; Masaki et al., 2012). Thus, it is worth examining the expression of TBP-2 in leiomyomas. However, there was no significant difference between the levels of TBP-2 mRNA of leiomyomas and matched myometrium (Fig. 4D). TBP-2 expression was not decreased in uterine leiomyomas in the present study. This may be consistent with the fact that leiomyomas are benign and not cancerous.

**Conclusion**

In the present study, we compared expressions of TRX-1 and TRX-1 related molecules in uterine leiomyomas and matched myometrium, although the expressions of molecules related to cancer proliferation increased in uterine leiomyomas compared with matched myometrium. However, the tumor suppressor TBP-2 expression was not altered. Thus, TRX-1 may be a target for uterine leiomyoma therapy. The detailed mechanisms involved in uterine leiomyoma need to be further studied.

**Authors’ roles**

B.J., H.P. and Z.L. substantially contributed to the conception and design of the project, while all authors contributed to acquisition, analysis and interpretation of data. B.J. wrote the article. All authors approved the final version of the article.

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**Conflict of interest**

None declared.

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