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RESEARCH

Association of Genetic Variations in *RTN4* 3'-UTR with Risk of Uterine Leiomyomas

Kui Zhang · Peng Bai · Shaoqing Shi · Bin Zhou · Yanyun Wang · Yaping Song · Li Rao · Lin Zhang

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Abstract This pilot case-control study was conducted to test the hypothesis that the TATC (rs71682890) and CAA (rs34917480) insertion/deletion polymorphisms of *RTN4* 3'-UTR are associated with the susceptibility to uterine leiomyoma (UL). The study recruited 286 premenopausal women with UL and 450 unrelated postmenopausal women not presenting the disease as control subjects. The polymorphisms of rs71682890 and rs34917480 were genotyped with the method of polymerase chain reaction polyacrylamide gel electrophoresis (PCR - PAGE). No statistically significant association was observed between the TATC insertion/deletion polymorphism and UL risk. However, increased UL risk was identified to be significantly associated with CAA insertion/deletion polymorphism in the recessive and co-dominant model. The present study provided evidence for the first time that CAA polymorphism in *RTN4* 3'-UTR, but not TATC polymorphism may be involved in susceptibility to UL.

Keywords *RTN4* · Uterine Leiomyoma (UL) · Polymorphism · Genetic susceptibility

Kui Zhang and Peng Bai have contributed equally to this work.

K. Zhang · P. Bai · L. Zhang (✉)
Department of Forensic Biology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, Sichuan 610041, People's Republic of China
e-mail: zhanglin@scu.edu.cn

S. Shi
Department of Immunology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, People's Republic of China

B. Zhou · Y. Wang · Y. Song
Laboratory of Molecular Translational Medicine, West China Institute of Women and Children's Health, Key Laboratory of Obstetric & Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu 610041, People's Republic of China

L. Rao
Department of Cardiology, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, People's Republic of China

Introduction

Uterine leiomyomas is the most common benign, solid, pelvic tumor in women, occurs in 20 %–40 % of women in their reproductive years [1]. Although UL is a very common condition, it has not received adequate attention from researchers, possibly due to being benign. However, various clinical problems such as pelvic pain, abnormal uterine bleeding, urinary frequency, infertility, recurrent pregnancy loss, and high risk of complications during pregnancy and childbirth are attributed to this disease [2–4]. It has been reported that UL forms the most common indication for hysterectomy [1]. Epidemiological survey revealed that 1 % of leiomyosarcoma was reported to arise in a preexisting leiomyoma [5]. Furthermore,UL consumed a significant amount of health care resources, and required expenditure of greater than 2.1 billion health care dollars annually in the United States [6].

Many causes for UL have been proposed, and it's clear that a hereditary predisposition to leiomyoma development exists. UL development has a genetic liability as evinced by monozygotic female twins being nearly twice as likely to be concordant for hysterectomy or for hospitalization due to UL as dizygotic twins [7]. But the genetics of UL pathogenesis is complex and largely unknown.

Nogo proteins, encoded by gene *reticulon-4* (*RTN4*), are myelin associated endoplasmic reticulum proteins, have been suggested to play an important role in apoptosis, especially in cancer cells [8–11]. Two genetic variations, TATC and CAA insertion/deletion in the 3'-untranslated region

(UTR) of *RTN4*, have been found. The 3'-UTR of eukaryotic genes was known to regulate gene expression, these genetic variations might induce abnormal regulation of *RTN4* gene expression [12] and cause diseases. The existing researches have revealed the associations of the above two genetic variations with diseases such as schizophrenia [13], dilated cardiomyopathy [14], congenital heart disease [15] and cervical squamous cell carcinoma [16]. However, the association of *RTN4* 3'-UTR TATC and CAA polymorphisms with UL remained unclear. Therefore, a hospital based case-control study was performed to test whether these genetic variations in *RTN4* 3'-UTR are associated with UL risk in Chinese Han women.

Material and Methods

Subjects

The present study was performed with the approval of the ethics committee of the Second University Hospital of Sichuan University and all the participants provided written informed consent. A hospital based case-control study was conducted including 286 unrelated premenopausal women with UL ranging in age from 26 to 49 year (mean ± SD, 47.02 ± 1.74) between July 2009 and May 2011 at the Second University Hospital of Sichuan University. The inclusion criteria were a diagnosis of uterine leiomyoma (confirmed by histopathology) and indication for surgical treatment. A group of control subjects including 450 healthy women ranging in age from 47 to 71 year (mean ± SD, 48.42 ± 2.48) was selected randomly from a routine health survey in the same hospital. The inclusion criteria for the control group were postmenopausal period and absence of uterine leiomyoma after clinical and ultrasonographic evaluation. Otherwise, we excluded from the control group with abnormal uterine bleeding and from the case group without surgical treatment. All subjects were Han women living in Sichuan Province of southwest China.

Genotyping

Genomic DNA of each individual was extracted from 200 μl of EDTA-anticoagulated peripheral blood samples by a DNA isolation kit from Bioteke (Peking, China). The procedure was performed according to instruction manual. Primers were established with the On-line software (http://frodo.wi.mit.edu/primer3/). PCR-PAGE was used to genotype the TATC and CAA insertion/deletion polymorphisms of *RTN4*. DNA fragments containing the polymorphisms were amplified by PCR using two primer pairs respectively. The primer sequences were: TATC-F 5′-cctgtcttgactgc catgtg-3′, TATC-R 5′-cggcaagactatctgcaaca-3′, and CAA-F 5′-tcaacatgaaatgccacaca-3′, CAA-R 5′-gcaaacaaacaa catttttgga-3′. PCR reaction was performed in a total volume of 25 μl, including 2.5 μl 10× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L dNTPs, 0.5 μmol/L each primer, 100 ng of genomic DNA and 1U of Taq DNA polymerase. The PCR conditions were 94 °C for 4 min, followed by 32 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C, with a final elongation at 72 °C for 10 min. Four microliters of PCR products were separated by a 6 % polyacrylamide gel and stained with 1.0 mg/ml argent nitrate. For the TATC polymorphism, allele $TATC_1$ yields a 146 bp band and allele $TATC_2$ yields a 150 bp band, and for the CAA polymorphism, allele CAA_1 yields a 124 bp band, and allele CAA_2 yields a 127 bp band. The genotypes were confirmed by DNA sequencing analysis (BigDye®Terminator v3.1 Cycle Sequencing Kits; Applied Biosystems, Foster City, CA). About 10 % of the samples were randomly selected to perform the repeated assays and the results were 100 % concordant.

Statistical Analysis

All data analyses were carried out by SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA). Genotype frequency of *RTN4* gene insertion/deletion polymorphisms were obtained by directed counting and Hardy–Weinberg equilibrium was evaluated by the chi-square test. Genotypic association tests in a case-control pattern assuming codominant, dominant, recessive, overdominant, or log-additive genetic models were performed using SNPstats [17]. Odds ratio (OR) and respective 95 % confidence intervals (CI) were reported to evaluate the effects of any difference between alleles, genotypes. A $P < 0.05$ was regarded as statistically significant in UL patients compared to healthy controls.

Results

Both TATC and CAA polymorphisms were successfully genotyped in 286 UL patients and 450 control subjects. Genotype distributions of these two polymorphisms in our cases and control subjects were consistent with the Hardy–Weinberg equilibrium. Allele frequencies of these two polymorphisms for 286 UL patients and 450 control subjects were shown in Table 1. As shown in Table 1, the allele frequencies of TATC and CAA polymorphisms in the UL patients were not significantly different from those of control subjects ($P = 0.279$, OR = 1.126, 95 % CI = 0.908 −1.396 for TATC; $P = 0.122$, OR = 0.840, 95 % CI = 0.674 −1.048 for CAA, respectively).

As shown in Table 2, significant association was observed for CAA polymorphism. Compared with (CAA_2/-CAA_2 + CAA_1/CAA_2) genotypes, CAA_1/CAA_1 genotype

Table 1 Information of selected polymorphisms in *RTN4* 3'-UTR among UL and controls

Polymorphisms	Allele	UL N = 286 (%)	Controls N = 450 (%)	OR (95 % CI)	*P* value
TATC	$TATC_1$	343(60.0)	565(62.8)	1.126(0.908-1.396)	0.279
	$TATC_2$	229(40.0)	335(37.2)		
CAA	CAA_1	206(36.0)	289(32.1)	0.840(0.674-1.048)	0.122
	CAA_2	366(64.0)	611(67.9)		

N corresponds to the number of individuals

carriers had a 1.79 fold increased UL risk ($P = 0.025$, 95 % CI = 1.08−2.94) in the recessive model. Compared with CAA_2/CAA_2 genotypes, CAA_1/CAA_1 genotype carriers had a 1.79 fold increased UL risk (95 % CI= 1.05−3.03), although not statistically significant ($P = 0.08$) in the co-dominant model. However, no statistically significant association was observed in the genetic models of the TATC polymorphism with UL risk.

Discussion

The present study was the first to investigate the association between the genetic variations in *RTN4* 3'-UTR and UL risk. Our results identified that homozygous carriers with CAA insertion significantly increased UL risk in the recessive model and in the codominant model. However, null results were observed in the TATC polymorphism.

The genetic pathogenesis of UL has been linked to hormone levels [18], enzymes [19] and cytogenetically visible genomic alterations. The cytogenetic alterations associated with UL risk varied and including large deletions on chromosome 7q [20, 21], 12q, 14q and 13q [22], but these occur at low frequency. Breakpoints in the more common cytogenetic rearrangements have exposed several candidate genes including *ORC5L*, *LHFPL3*, and *PCOLCE* [23]. A significant fraction of UL without visible cytogenetic changes harbor submicroscopic genomic rearrangements which may in turn contribute to transformation of normal myometrial tissue into leiomyomas [24] has been identified

Table 2 Genotype frequencies of selected polymorphisms in *RTN4* 3'-UTR among UL and controls and their association with UL risk

Genetic model	Genotype	UL	Controls	Logistic regression#	
		N = 286 (%)	N = 450 (%)	OR (95 % CI)	*P* value
TATC					
Codominant	$TATC_1/TATC_1$	111(38.8)	182(40.4)	1.00 (reference)	0.38
	$TATC_1/TATC_2$	121 (42.3)	201(44.7)	1.15(0.81−1.63)	
	$TATC_2/TATCA_2$	54(18.9)	67 (14.9)	0.84 (0.53−1.33)	
Dominant	$TATC_1/TATC_1$	111 (38.8)	182(40.4)	1.00 (reference)	0.76
	$TATC_1/TATC_2$ + $TATC_2/TATCA_2$	175 (61.2)	268(59.6)	1.05 (0.76−1.46)	
Recessive	$TATC_1/TATCA_1$+$TATC_1/TATCA_2$	232(81.1)	383(85.1)	1.00 (reference)	0.25
	$TATC_2/TATC_2$	54(18.9)	67(14.9)	0.78 (0.51−1.19)	
Overdominant	$TATC_1/TATCA_1$ + $TATC_2/TAT_2$	165 (57.7)	249 (55.3)	1.00 (reference)	0.24
	$TATC_1/TATC_2$	121 (42.3)	201 (44.7)	1.21 (0.88−1.67)	
Log−additive				0.96 (0.76−1.20)	0.69
CAA					
Codominant	CAA_2/CAA_2	120 (42.0)	203 (45.1)	1.00 (reference)	0.08
	CAA_1/CAA_2	126(44.1)	205(45.6)	098(0.70−1.38)	
	CAA_1/CAA_1	40 (14.0)	42 (9.3)	**1.79(1.05−3.03)**	
Dominant	CAA_2/CAA_2	120 (42.0)	203 (45.1)	1.00 (reference)	0.43
	CAA_1/CAA_2 + CAA_1/CAA_1	166 (58.0)	247 (54.9)	0.88 (0.64−1.21)	
Recessive	CAA_2/CAA_2 + CAA_1/CAA_2	246 (86.0)	408 (90.7)	1.00 (reference)	**0.025**
	CAA_1/CAA_1	40 (14.0)	42 (9.3)	**1.79(1.08−2.94)**	
Overdominant	CAA_1/CAA_1 + CAA_2/CAA_2	160 (55.9)	245 (54.4)	1.00 (reference)	0.53
	CAA_1/CAA_2	126 (44.1)	205 (45.6)	1.11(0.81−1.53)	
Log-additive				0.82 (0.64−1.04)	0.097

#adjusted by age

N corresponds to the number of individuals

Boldfaced values indicate a significant difference at the 5 % level

recently. However, genetic pathogenesis of UL is complex and still largely unknown.

Reticulons is the only molecular so far to participate in all three apoptosis signaling pathways, including death receptor mediated pathway, mitochondrial pathway,and ER-stress pathway [25]. Nogo protein, as a reticulon family protein, contains two transmembrane domains and a C-terminal double lysine endoplasmic reticulum retrieval motif. Derived from differential splicing and varied promoter usage, the *RTN4* gene produces 3 major isoforms, namely Nogo-A, Nogo-B and Nogo-C [26, 27]. Nogo-A, mainly expressed in the central nervous system, has been identified as an inhibitor of axonal regeneration, Nogo-B is found in most tissues, while Nogo-C is highly expressed in skeletal muscles [28].

Recent studies indicated that Nogo protein was apoptosis-inducing protein, and involved in the process of apoptosis through some classical apoptotic signal pathways [8, 9, 11, 29]. Nogo-C was reported to inhibit SMMC7721 cells growth and promote its apoptosis by transferring mutant p53 protein from nucleus to cytoplasm and decreasing c-Fos, Hsp70 protein expression [29]. And also, *RTN4* was reported to express in HEK293 confers apoptosis by inducing caspase-3 and p53 activation through the JNK-c-Jun-dependent pathway [8]. Overproduction of Nogo-B in the graft vein could result in reduced neointimal hyperplasia, the mechanism of which involved increased smooth muscle cell apoptosis induced by activation of the JNK/p38 MAPK pathway [30]. Nogo proteins induce apoptosis in various cancer cells when overexpressed, whereas normal cells are relatively resistant to Nogo protein-dependent apoptosis [31].

The human *RTN4* gene, mapped to chromosome 2p12-14, spans over 75 kb, consists of nine axons and eight introns, and this gene includes a 244 bp 5'-UTR and an over 1 kb 3'-UTR [26, 27]. The TATC and CAA polymorphisms of *RTN4* 3'-UTR are located at 4068−4071 and 4548−4554 in AY102279, respectively. The 5'-UTR and 3'-UTR of eukaryotic mRNAs have been proved to regulate the expression of gene and the 3'-UTR is involved in the regulation of translation initiation, mRNA stability and subcellular localization. The functional role of TATC and CAA polymorphisms in *RTN4* 3'-UTR remained unknown and it didn't match any known 3'-UTR functional motifs. Few studies have revealed the association between these genetic variants and diseases such as schizophrenia [13], dilated cardiomyopathy [14], congenital heart disease [15] and cervical squamous cell carcinoma [16]. In the current study, the homozygous carriers of CAA insertion allele were revealed to be associated with high UL risk; this result implied the potent association of genetic variations in *RTN4* 3'-UTR with UL risk.

The investigation might have some limitations. Our study was limited by relatively small sample size, which weakened our ability to solidify statistic associations. Further studies in ethnic different population and with a larger size of samples could help to confirm the true significance of the association between these polymorphisms and the susceptibility to UL.

In conclusion, for the first time, this study have demonstrated that CAA polymorphism in *RTN4* 3'-UTR, but not TATC polymorphism is associated with increased UL risk. Due to limitations of sample size, further studies with large scaled participants are needed to confirm the underlying association.

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References

1. Duhan N (2011) Current and emerging treatments for uterine myoma - an update. Int J Womens Health 3:231–241
2. Hart RKY, Yeong CT, Seed P, Taylor A, Braude P (2001) A prospective controlled study of the effect of intramural uterine fibroids on the outcome of assisted conception. Hum Reprod 16:2411–2417
3. Ptacek TSC, Walker CL, Sell SM (2007) Physical mapping of distinct 7q22 deletions in uterine leiomyoma and analysis of a recently annotated 7q22 candidate gene. Cancer Genet Cytogenet 174:116–120
4. Morgan Ortiz FPRB, Elorriaga García E, Báez Barraza J, Quevedo Castro E, Peraza Garay Fde J (2011) Uterine leiomyomas during pregnancy and its impact on obstetric outcome. Ginecol Obstet Mex 79(8):467–473
5. Lee EJ, Kong G, Lee SH, Rho SB, Park CS, Kim BG, Bae DS, Kavanagh JJ, Lee JH (2005) Profiling of differentially expressed genes in human uterine leiomyomas. Int J Gynecol Cancer 15 (1):146–154. doi:10.1111/j.1048-891x.2005.15016.x
6. Flynn MJM, Datta S, Myers E (2006) Health care resource use for uterine fibroid tumors in the United States. Am J Obstet Gynecol 195(4):955–964
7. Luoto RKJ, Rutanen EM, Taipale P, Perola M, Koskenvuo M (2000) Heritability and risk factors of uterine fibroids–the Finnish Twin Cohort study. Maturitas 37(1):15–26
8. Chen Y, Tang X, Cao X, Chen H, Zhang X (2006) Human Nogo-C overexpression induces HEK293 cell apoptosis via a mechanism that involves JNK-c-Jun pathway. Biochem Biophys Res Commun 348(3):923–928. doi:10.1016/j.bbrc.2006.07.166
9. Shimakage MIN, Ohshima K, Kawahara K, Oka T, Yasui K, Matsumoto K, Inoue H, Watari A, Higashiyama S, Yutsudo M (2006) Down-regulation of ASY/Nogo transcription associated with progression of adult T-cell leukemia/lymphoma. Int J Cancer 119(7):1648–1653
10. Jung TYJS, Lee KH, Cao VT, Jin SG, Moon KS, Kim IY, Kang SS, Kim HS, Lee MC (2011) Nogo-A expression in oligodendroglial tumors. Neuropathology 31(1):11–19

11. Yicun Chen XT, Zhang X, Zhuang L (2009) New mutations of Nogo-C in hepatocellular carcinoma. Mol Biol Rep 36:377–380
12. Novak G, Tallerico T (2006) Nogo A, B and C expression in schizophrenia, depression and bipolar frontal cortex, and correlation of Nogo expression with CAA/TATC polymorphism in 3'-UTR. Brain Res 1120(1):161–171. doi:10.1016/j.brainres.2006.08.071
13. Novak G, Kim D, Seeman P, Tallerico T (2002) Schizophrenia and Nogo: elevated mRNA in cortex, and high prevalence of a homozygous CAA insert. Brain Res Mol Brain Res 107(2):183–189
14. Zhou B, Rao L, Li Y, Gao L, Li C, Chen Y, Xue H, Liang W, Lv M, Song Y, Peng Y, Zhang L (2009) The association between dilated cardiomyopathy and RTN4 3'UTR insertion/deletion polymorphisms. Clin Chim Acta 400(1–2):21–24. doi:10.1016/j.cca.2008.09.028
15. Chen Y, Zhou B, Li H, Peng Y, Wang Y, Rao L (2011) Analysis of RTN4 3'UTR insertion/deletion polymorphisms in ventricular septal defect in a Chinese Han population. DNA Cell Biol 30(5):323–327. doi:10.1089/dna.2010.1116
16. Shi S, Zhou B, Wang Y, Chen Y, Zhang K, Wang K, Quan Y, Song Y, Rao L, Zhang L (2012) Genetic variation in RTN4 3'-UTR and susceptibility to cervical squamous cell carcinoma. DNA Cell Biol 31(6):1088–1094. doi:10.1089/dna.2011.1548
17. Sole X, Guino E, Valls J, Iniesta R, Moreno V (2006) SNPStats: a web tool for the analysis of association studies. Bioinformatics 22:1928–1929
18. Wei TGA, Qian HR, Su C, Helvering LM, Kulkarini NH, Shou J, N'Cho M, Bryant HU, Onyia JE (2007) DNA microarray data integration by ortholog gene analysis reveals potential molecular mechanisms of estrogen-dependent growth of human uterine fibroids. BMC Womens Health 7:5
19. Hiroshi Ishikawa SR, Demura M, Rademaker AW, Kasai T, Inoue M, Usui H, Shozu M, Bulun SE (2009) High aromatase expression in uterine leiomyoma tissues of African-American women. J Clin Endocrinol Metab 94:1752–1756
20. Ishwad CSFR, Hanley K, Davare J, Meloni AM, Sandberg AA, Surti U (1997) Two discrete regions of deletion at 7q in uterine leiomyomas. Genes Chromosomes Cancer 19:156–160
21. Xing YPPW, Morton CC (1997) The del(7q) subgroup in uterine leiomyomata: genetic and biologic characteristics. Further evidence for the secondary nature of cytogenetic abnormalities in the pathobiology of uterine leiomyomata. Cancer Genet Cytogenet 98(69–74):69
22. Meloni AMSU, Sandberg AA (1991) Deletion of chromosome 13 in leiomyomas of the uterus. Cancer Genet Cytogenet 53:199–203
23. Jennelle C, Hodge KTC, Huyck KL, Somasundaram P, Panhuysen CIM, Stewart EA, Morton CC (2009) Uterine leiomyomata and decreased height: a common HMGA2 predisposition allele. Hum Genet 125(3):257–263
24. Bowden WSJ, Kovanci E, Rajkovic A (2009) Detection of novel copy number variants in uterine leiomyomas using high-resolution SNP arrays. Mol Hum Reprod 15(9):563–568
25. Yaqin Chen SZ, Xiang R (2010) RTN3 and RTN4: candidate modulators in vascular cell apoptosis and atherosclerosis. J Cell Biochem 111:797–800
26. Oertle T, Huber C, van der Putten H, Schwab ME (2003) Genomic structure and functional characterisation of the promoters of human and mouse nogo/rtn4. J Mol Biol 325(2):299–323
27. Yang J, Yu L, Bi AD, Zhao SY (2000) Assignment of the human reticulon 4 gene (RTN4) to chromosome 2p14–2p13 by radiation hybrid mapping. Cytogenet Cell Genet 88(1–2):101–102
28. GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403(6768):439–444. doi:10.1038/35000226
29. Chen YC, Lu DD, Cao XR, Zhang XR (2005) RTN4-C gene expression in hepatocellular carcinoma and its influence on SMMC7721 cell growth and apoptosis. Yi Chuan Xue Bao 32 (9):891–897
30. Zheng H, Xue S, Lian F, Wang YY (2011) A novel promising therapy for vein graft restenosis: overexpressed Nogo-B induces vascular smooth muscle cell apoptosis by activation of the JNK/p38 MAPK signaling pathway. Med Hypotheses 77(2):278–281. doi:10.1016/j.mehy.2011.04.035
31. Watari A, Yutsudo M (2003) Multi-functional gene ASY/Nogo/RTN-X/RTN4: apoptosis, tumor suppression, and inhibition of neuronal regeneration. Apoptosis 8(1):5–9