SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 17: 5435-5439, 2018

Novel TRERF1 mutations in Chinese patients with ovarian endometriosis

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Received October 11, 2017; Accepted December 20, 2017

DOI: 10.3892/mmr.2018.8510

Abstract. Endometriosis is an estrogen-dependent precancerous lesion exhibiting frequently perturbed level of steroid hormones and transcriptional-regulating factor 1 (TRERF1) has a crucial role in the production of steroid hormones including estrogen. Endometriosis has previously been revealed to be a precancerous lesion that harbors somatic mutations in cancer-associated genes. Therefore, the authors of the present study hypothesize that TRERF1 aberrations may be involved in the development of endometriosis. In the present study, endometriotic lesions and paired blood samples from 92 individuals with ovarian endometriosis were analyzed for the potential presence of TRERF1 mutations by sequencing the entire coding region and the corresponding intron-exon boundaries of the TRERF1 gene. Two heterozygous missense somatic mutations [c.3166A>C (p.K1056Q) and c.3187 G>A (p.G1063R)] in the TRERF1 gene were identified in two out of 92 ectopic endometria (2.2%), to the best of our knowledge, these mutations have not been previously reported. From the two samples with TRERF1 mutations, one sample was from a 42-year-old patient also diagnosed with uterine leiomyoma and the other mutation was identified in a 36-year-old woman exhibiting no other apparent gynecological conditions. The evolutionary conservation analysis and *in silico* prediction of these TRERF1 mutations suggested that they may be pathogenic. To the best of our knowledge, the present study was the first to identify 2 novel, potentially 'disease-causing' TRERF1 somatic mutations in the endometriotic lesions in 2 out of 92 patients with ovarian endometriosis; therefore, TRERF1 mutations may be involved in the pathogenesis of ovarian endometriosis.

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Key words: transcriptional-regulating factor 1, mutations, ovarian endometriosis, Chinese

Introduction

Endometriosis is an estrogen-dependent chronic disorder that affects between 5 and 10% of women at reproductive age (1,2). It is characterized by chronic pelvic pain and subfertility or infertility, and affects the quality life of the affected individuals (3-5). Despite the progress made in elucidating the molecular etiology of endometriosis, due to heterogeneity of this disorder, there are individuals with unknown molecular/genetic alterations which are affected by endometriosis (6-9).

Endometriosis has long been considered to be closely associated with the development of ovarian endometrioid and clear cell carcinoma, and has been previously proposed to be a precancerous lesion (10-12). Previous studies further supported this hypothesis as an increasing number of genetic alterations in oncogenes and tumor suppressor genes, such as KRAS proto-oncogene GTPase, tumor protein p53, phosphatase and tensin homolog, breast cancer type 2 susceptibility protein and protein phosphatase 2 scaffold subunit Aα, have been identified in patients with endometriosis (13-15).

Transcriptional regulating factor 1 (TRERF1) acts as a zinc-finger transcriptional regulatory protein and modifies the expression of cholesterol side-chain cleavage enzyme (P450scc) (16,17). Endometriosis is frequently characterized by perturbed levels of estrogen (18,19) and diverse somatic mutations in multiple genes (14). TRERF1 may regulate the expression of P450scc, thus affecting the conversion of cholesterol to pregnenolone, which is the first and rate-limiting step in the synthesis of the steroid hormones, such as estrogen (20). The authors of the present study hypothesize that certain TRERF1 aberrations, including gene mutations, may contribute to the development of endometriosis. To verify this hypothesis, samples were collected from 92 patients with ovarian endometriosis and the entire coding region and corresponding intron-exon boundaries of TRERF1 were sequenced to analyze the potential presence of TRERF1 mutations.

Materials and methods

Samples. The ectopic endometria and paired blood samples were obtained from a total of 92 patients with ovarian endometriosis who underwent surgical resection in Jiangxi Provincial Maternal and Child Health Hospital between June 2013 and

July 2014. Tissue and blood samples were stored at -80˚C immediately following collection.

Clinical data. Clinical data was determined for individuals with ovarian endometriosis, including the following information: Age of diagnosis, age at the time of menarche, the serum levels of estrogen (E2), progesterone (P), cancer antigen 125 (CA125), thyroid stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4), carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and squamous cell carcinoma antigen (SCCA) is presented in Table I. The levels for the aforementioned factors were determined according to the previously described protocols (21).

Compliance with ethical standards. The present study was approved by the Ethics Committee at the Jiangxi Provincial Maternal and Child Health Hospital (Nanchang, China). All procedures were performed according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each patient prior to this study.

DNA isolation, polymerase chain reaction (PCR) amplification and DNA sequencing. Genomic DNA (gDNA) was isolated from tissues and paired blood samples using a TIANamp Genomic DNA kit (cat no. DP304; Tiangen Biotech Co., Ltd., Beijing, China). The quantity and quality of the isolated gDNA was assessed by SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 260 nm and 1.5% agarose gel electrophoresis with ethidium bromide staining for visualization, respectively. The entire coding sequence of the TRERF1 gene was amplified and sequenced to analyze potential somatic mutations in 92 ovarian endometriosis samples. A total of 50 ng DNA from each sample was amplified using PCR in a final volume of 30 μl containing 1.0 U of rTaq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China), 3 μl 10X PCR buffer (Takara Biotechnology Co., Ltd. Dalian, China), 1.0 mM $MgCl_2$, 200 μM dNTPs (Takara Biotechnology Co., Ltd., Dalian, China), 1.5 μM each primer in a Thermal Cycler 2720 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following thermocycling conditions were used for the PCR: Initial denaturation for 5 min at 94˚C, 35 cycles of 94˚C for 30 sec, 52-62˚C for 30 sec, and 72˚C for 30 sec, and a final extension at 72˚C for 8 min. The purification of the amplified PCR products was performed using TIANgel Midi Purification kit (Tiangen Biotech Co., Ltd. Beijing, China). The purified PCR products were subjected to a sequencing reaction using ABI PRISM® BigDye® Terminator v3.1 cycle Sequencing kit (Thermo Fisher Scientific, Inc.) with ABI Prism 3730 DNA sequencer (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequencing electropherograms were analyzed using DNASTAR software version 4.0 (DNASTAR, Inc., Madison, WI, USA). Somatic mutations were confirmed by sequencing DNA from paired blood samples. The PCR primer sequences for the entire coding sequence of the TRERF1 gene are listed in Table II.

Evolutionary conservation analysis. To evaluate the role of the identified TRERF1 somatic mutations, evolutionary conservation analysis of the TRERF1 protein sequence was performed. The amino acid sequences of 19 vertebrate species were obtained from GenBank database and subjected to the evolutionary conservation analysis of TRERF1, including: *Homo sapiens* (accession no. NP_277037.1), *Pan troglodytes* (accession no. XP_016810987), *Macaca mulatta* (accession no. XP_014991838), *Cercocebus atys* (accession no. XP_011928290), *Mus musculus* (accession no. NP_001091092), *Rattus norvegicus* (accession no. NP_001101669), *Bos taurus* (accession no. XP_010816556), *Canis lupus familiaris* (accession no. XP_013973919), *Camelus dromedarius* (accession no. XP_010977853), *Equus asinus* (accession no. XP_014686382), *Panthera pardus* (accession no. XP_019312677), *Mustela putorius furo* (accession no. XP_012917709), *Microcebus murinus* (accession no. XP_012628645), *Pteropus vampyrus* (accession no. XP_011352924), *Gallus gallus* (accession no. XP_015139339), *Gavialis gangeticus* (accession no. XP_019371939), *Manis javanica* (accession no. XP_017504614), *Xenopus tropicalis* (accession no. XP_002934726) and *Nanorana parkeri* (accession no. XP_018413822). The alignment of sequences was carried out by ClusterW method using MEGA version 4.0 (22).

Table I. Association of transcriptional-regulating factor 1 mutations with clinical characteristics in the 92 individuals with ovarian endometriosis.

Feature	Wild type (n=90)	Mutant type (n=2)	P-value
Age (years)	33.54±7.45	39.00±4.24	0.31
Age of menarche (years)	13.68±1.38	14±2.83	0.75
E2 (pg/ml)	126.65±99.15	84.61±61.51	0.54
P (ng/ml)	1.54±3.68	0.91±0.49	0.81
CA125 (μ/ml)	103.38±191.13	57.03±14.60	0.73
TSH (mIU/ml)	2.60±1.25	1.90±0.43	0.31
FT3 (pg/ml)	3.05±0.37	3.00±0.04	0.87
FT4 (ng/dl)	1.29±0.13	1.30±0.06	0.56
CEA (ng/ml)	1.16±0.42	0.96±0.11	0.36
AFP (ng/ml)	2.53±1.62	3.47±0.77	0.43
SCC (ng/ml)	1.48±1.01	1.68±1.28	0.65

E2, estrogen; P, progesterone; CA125, cancer antigen 125; TSH, thyroid stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; CEA, carcinoembryonic antigen; AFP, α-fetoprotein; SCCA, squamous cell carcinoma antigen.

Bioinformatics prediction of TRERF1 mutations. The online bioinformatics programs, PolyPhen-2 (23,24) and MutationTaster version 2 (25) were used to predict the potential disease-causing roles for the identified TRERF1 mutations, using the instructions of the programs.

Statistical analysis. Student's t-test was used to compare the potential association between nominal variables referring to TRERF1 mutations, and continuous variables were

Table II. Polymerase chain reaction primer sequences for the mutation analysis of transcriptional-regulating factor 1 gene.

Exon	Primer sequence (5'-3')		Annealing temperature (˚C)	Amplicon length (bp)
	Forward	Reverse		
5-1	GACGTCTCCTCACCACAGTG	CCAGTGAAACCAGGGTGAGG	55	891
5-2	TCAGCAGTGATGGATGGAGC	CTGACCCTGTAGCACACTGG	60	981
6	GGTGGTCCCAAGTCAAGGAG	CACCCCAGAAAATCCTCCCC	57	312
7	CTCTAAAGGGCACTGGGGTG	CAAGCAGCACACGACCTAGA	50	355
8	AAGTGCATCCCCCTTGTGAG	GGGTAGGGTTCCCAATGTGG	52	521
9	CAACCAGAACTCGCTTTGCC	GTCCCAGGACTTTACCCAGC	52	620
10	CACCATACTCCACCCAGCTC	AGGGCTTCATGCTTTGACCA	52	446
11, 12	CAGTGAAAAGGCCACGTGTG	CCTACCCACCGAGAGAAGGA	55	697
13	TCCCTCTGGGTTTCCTTCCA	CACAACCGAACATGCAAGCA	55	279
14	GAACCCAGGTGTCAGAGCTC	CCAGCGAGTGTGGAAGACAT	50	393
15	GGTAAGGACAGGCGTGTGAA	GGCTATCTTGGCAGCAAAGC	52	382
16	TCCTAAGCATCCGGAGACCA	CCCTCTGCCAAACTGTGACT	57	519
17	ACAGGATCTGTGGTTGTGGT	TCCCATAGAGCGACTACCCA	62	457
18	AGGAGGTCCTAGAAGCCGAG	TTATTTATTCCCCCAACCCCC	57	663

bp, base pair.

compared using the Mann-Whitney method. P-values were 2-tailed and P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

TRERF1 mutations in ovarian endometriosis. In the present study, the coding sequence and the corresponding intron-exon boundaries of the TRERF1 gene in the ectopic endometria from 92 individuals with ovarian endometriosis were sequenced. Two heterozygous missense somatic mutations in TRERF1 [NM_033502; c.3166A>C (p.K1056Q) and c.3187 G>A (p.G1063R)] located in exon 17 were identified in 2 out of 92 ectopic endometria (2.2%) samples. The somatic status of these mutations was confirmed by sequencing of the respective paired blood samples (Fig. 1). From the two samples with TRERF1 mutations, one sample was from a 42-year-old diagnosed with uterine leiomyoma, and the other mutation carrier was a 36-year-old woman exhibiting no other apparent gynecological conditions. No TRERF1 mutations were identified in the remaining 90 samples of ovarian endometriosis. Furthermore, the two novel mutations were not identified in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) (26) and dbSNP database (https://www.ncbi.nlm.nih.gov/snp).

Association between TRERF1 mutations and clinical data. The potential association between TRERF1 mutations and the available clinical data was analyzed using SPSS software. Therefore, no association between TRERF1 mutations and clinical characteristics was identified (Table I).

Evolutionary conservation analysis of TRERF1 mutations. Evolutionary conservation analysis revealed that the mutated amino acids p.K1056 and p.G1063 led to alterations of highly conserved amino acid sequences in vertebrate species (Fig. 2).

Pathogenic potential of TRERF1 mutations. The TRERF1 mutations were predicted by MutationTaster online program and the two TRERF1 mutations (p.K1056Q and p.G1063R) were predicted to be 'disease-causing', with a score of 125 (p.K1056Q) and 53 (p.G1063R), where the mutations were considered as 'disease-causing' when the mutation frequency was >0.01 and the mutation was not present in the known single-nucleotide polymorphisms (SNPs) database in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/snp/). Additionally, the two mutations were also predicted to be 'probably damaging' by PolyPhen-2, with a score of 0.999 (sensitivity, 0.14; specificity, 0.99) for p.K1056Q mutation and a score of 1.000 (sensitivity: 0.00; specificity: 1.00) for p.G1063R mutation, where the mutations were considered as 'probably damaging' when the prediction score value was >0.05.

Discussion

Previous studies have suggested that the balance between estrogen and progesterone production is frequently perturbed in endometriosis (18,19) and that endometriosis is a potential precancerous lesion harboring multiple somatic mutations in certain cancer-associated genes (10,11,13-15). It remains to be determined whether endometriosis harbors mutations in genes involved in the regulation of production of steroid hormones. Considering that TRERF1 has a role in the production of steroid hormones, including estrogen and progesterone (16,20),

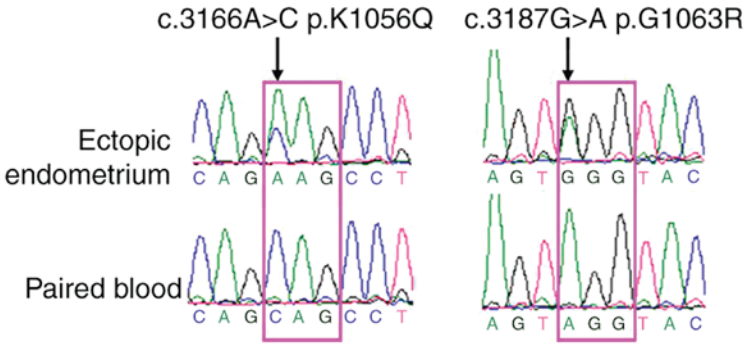

Figure 1. Sequencing electropherograms of TRERF1 mutations in ectopic endometria and the corresponding blood samples. The arrows indicate the locations of the mutations.

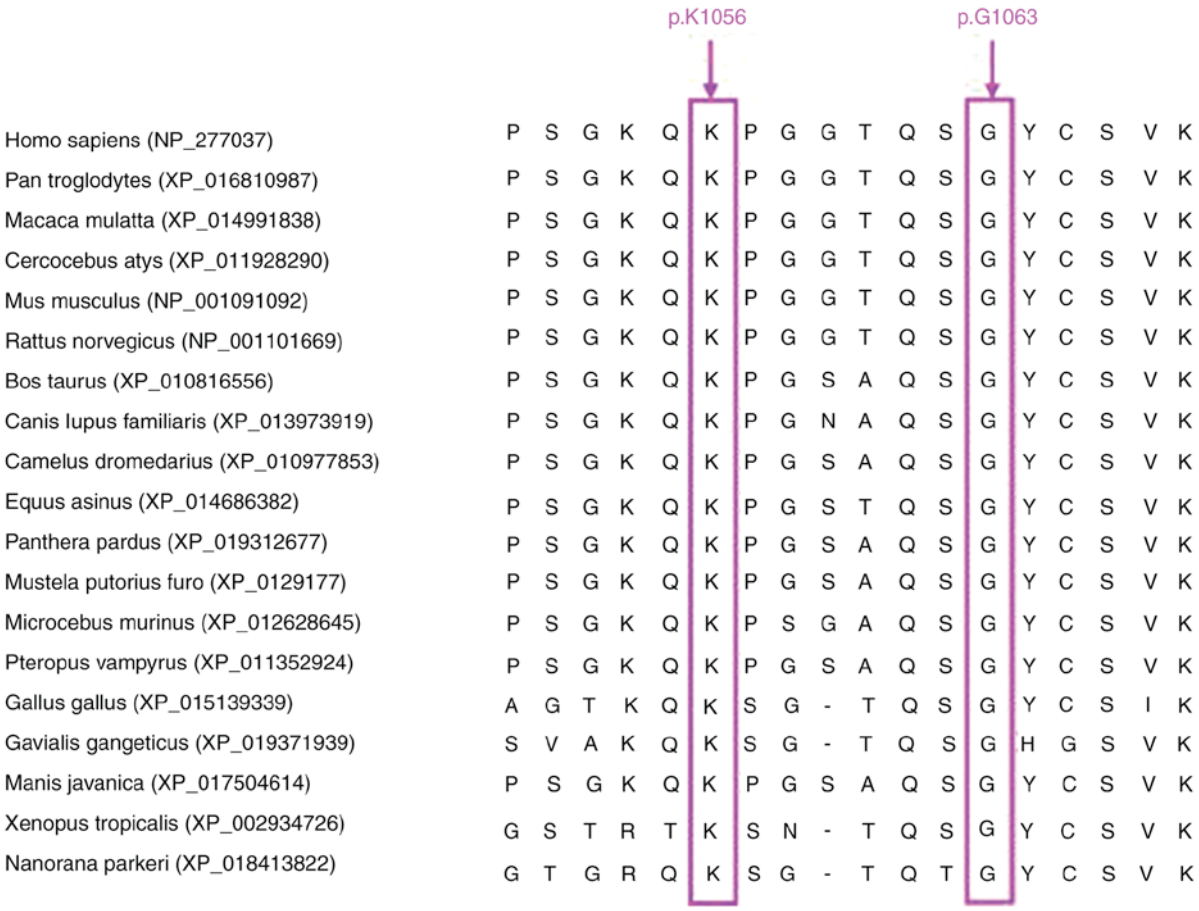

Figure 2. Evolutionary conservation analysis results of TRERF1 p.K1056Q and p.G1063R mutations in 19 different vertebrate species.

the authors of the present study hypothesized that TRERF1 may harbor mutations in endometriosis.

In the present study, two heterozygous somatic mutations in the TRERF1 gene were identified in two out of 92 tissue samples with ovarian endometriosis. To the best of our knowledge, both mutations were not previously reported and are located in exon 17. One individual carrying the somatic mutation of TRERF1 gene was diagnosed with uterine leiomyoma while the other exhibited no other apparent gynecological conditions. In addition, no association between TRERF1 mutation and the collected clinical data was observed in the samples included in the present study, including age at diagnosis, age of menarche, the levels of serum E2, P, CA125, TSH, FT3, FT4, CEA, AFP and SCCA. The results of the statistical analysis should be treated with caution due to small sample size in the TRERF1 mutation group (n=2). The evolutionary conservation analysis suggested that both the p.K1056 and p.G1063 residues were evolutionarily highly conserved in a number of vertebrate species. Furthermore, the two TRERF1 mutations were predicted to be 'disease-causing' and 'probably damaging' according to MutationTaster and PolyPhen-2 prediction programs, respectively. However, whether these TRERF1 mutations have a role in the development of ovarian endometriosis remains to be confirmed.

The mutation frequency of TRERF1 among patients with ovarian endometriosis was 2.2% (2/92). Previous studies aiming to determine the somatic mutation profiles in ovarian endometriosis did not identify any somatic mutations in the TRERF1 gene in endometriotic lesions from 16 patients with ovarian endometriosis (14) and 27 patients with deep infiltrating endometriosis (15). The authors of the present study hypothesize that the relatively small sample sizes analyzed in the prior studies may have prevented identification of the potential rare somatic mutations (14,15).

In conclusion, the present study identified somatic mutations in TRERF1 (p.K1056Q and p.G1063R) in ovarian endometriosis and the mutation frequency was 2.2% (2/92). *In silico* prediction suggested that the two somatic mutations may be 'disease-causing'. Future functional assays should be performed to confirm the pathogenic roles of the TRERF1 mutations, which may elucidate the underlying mechanism of ovarian endometriosis.

Acknowledgements

The present study was supported by a grant from the Natural Science Foundation of Jiangxi Province (grant no. 20151BAB205012).

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