

Cyclin D1 G870A polymorphism: Association with uterine leiomyoma risk and *in silico* analysis

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Abstract. Uterine leiomyoma (UL) is the most common benign tumor causing considerable morbidity during the reproductive years in women. Cyclin D1 (CCND1) is a cell cycle regulatory protein that is required for the G1 phase, and increased expression levels of this protein may affect tumorigenesis. The present study aimed to assess the possible effect of the CCND1 G870A polymorphism on UL susceptibility. A total of 154 women with UL and 197 healthy women who were age-, body mass index (BMI)- and ethnicity-matched were genotyped for the CCND1 G870A (rs9344) polymorphism using the polymerase chain reaction-restriction fragment length polymorphism method. The effects of G870A transition on the structure of mRNA and proteins of CCND1 was evaluated using bioinformatics tools. The frequency of the CCND1 870AA genotype was significantly higher in women with UL compared with the control subjects, and the risk of UL was 1.4-fold higher in women with the AA genotype when compared with the GG genotype before and after adjusting for age, BMI, and ethnicity [odds ratio (OR), 1.4; 95% confidence interval (CI), 1.1-2 (P=0.02)]. The frequency of CCND1 870GA genotype was not significantly different between the two groups. The frequency of the CCND1 870A allele was significantly higher in the women with UL when compared with the control subjects (57 vs. 48%; P=0.02). The *in silico*

analysis revealed that the G870A transition may fundamentally alter the structure of the CCND1-mRNA. Thus, the CCND1 870AA genotype was associated with UL susceptibility in a sample of women from the southeast of Iran.

Introduction

Uterine leiomyomas (ULs) are benign tumors arising from smooth muscle cells of the uterine myometrium. ULs are characterized by the high smooth muscle cell (SMCs) proliferation and marked quantities of extracellular matrix proteins, predominantly collagen type I and III (1). UL development is considered a multistep and multi-factorial process, for which growth factors, cytokines, and ovarian steroid hormones are key in its formation and development (2). Different studies have shown that UL originate from somatic mutation in myometrial cells, which results in progressive loss of growth regulation (3). These tumors proliferate from a single progenitor cell and grow as abnormal clones of cells. ULs are monoclonal, and different leiomyomas arising within one uterus are not clonally linked (4). In addition, different rates of growth are due to chromosomal abnormalities within individual tumors (5). The occurrence of multiple ULs indicates a genetic predisposition for UL formation. There is a ~2.5-fold increased risk of developing UL in first-degree relatives of women with these types of tumor (6).

Abnormal cell proliferation is an important step in tumor development. There are various proteins in the cell cycle pathway that affect cell proliferation and tumorigenesis. Among these proteins, cyclins are particularly important in this process (7). Cyclins are a family of proteins that affect cell progression through the cell cycle by triggering cyclin-dependent kinase (CDK) enzymes. Cyclin D1 (CCND1) is a key regulatory protein that binds to CDK4 and CDK6 enzymes, and functions during the transition from the G1 to the S phase (8). Increased expression of the CCND1 gene may disrupt normal cell cycle control, and prompt cells to

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transition from the G₁/S checkpoint of the cell cycle resulting in tumor formation (8). Various studies have demonstrated that overexpression of the CCND1 protein is associated with cell proliferation and different types of cancer, including endometrial (9), cervical (10), lung (11) and breast (12) cancer.

Betticher *et al* (13) described a G to A single nucleotide polymorphism (G870A) at the exon4/intron4 splicing region of the *CCND1* gene. The A allele has a longer half-life than the G allele, which is hypothesized to increase CCND1 levels resulting in the proliferation of abnormal cells and the escape of these cells from apoptosis (13). To the best of our knowledge, there is only one published report that has indicated the effect of the *CCND1* G870A polymorphism on UL susceptibility in obese Korean women (14), although there are different reports regarding the association between CCND1 gene polymorphisms, and tumors and cancers.

Thus, the present study aimed to assess the possible association between the *CCND1* G870A polymorphism and UL in a southeastern Iranian population.

Materials and methods

Study population. A total of 154 Iranian women (aged 37.9±8.9 years) with UL in their pre-menopause stage who had undergone myomectomy or hysterectomy and 197 Iranian healthy women (aged 37.5±5.5 years) in their pre-menopause stage were enrolled in the study. All individuals were recruited from the Department of Obstetrics and Gynecology of Ali-ebn-Abitaleb Educational Hospital of Zahedan University of Medical Sciences (Zahedan, Iran) from July 2012 to October 2013. UL was confirmed pathologically in all of the women. The control group was selected from women that were referred for routine yearly check-ups and undergone the Pap smear test. The UL and control groups were matched with respect to age, body mass index (BMI), and ethnicity (Fars or Balouch). Upon sonography or examination, the participants in the control group exhibited no evidence of UL and their Pap smear test was negative. Women with systemic disease and history of malignancy were excluded from the present study. The participants provided their informed consent prior to participating in the study and the study protocol was approved by the Ethics Committee of Zahedan University of Medical Sciences (Zahedan, Iran) and conducted in accordance with the Declaration of Helsinki.

Genomic DNA extraction and genotyping. Venous blood (2 ml) was drawn from each participant. Genomic DNA was isolated from 200 µl EDTA-treated whole blood using the salting out phenol chloroform method and stored at 20°C until analysis. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed for genotyping the *CCND1* G870A polymorphism.

The 167-bp fragment containing the *CCND1* G870A polymorphism (rs9344) was amplified using primers as previously described (13). The PCR conditions were as follows: 6 min at 95°C followed by 30 cycles of denaturation at 96°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 6 min. *NciI* restriction enzyme (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) was used to digest the PCR products at 37°C overnight.

Table I. Clinical and demographic characteristics of the UL women and control group subjects.

Parameter	UL women (n=154)	Control subjects (n=197)	P-value
Age (years)	37.9±8.9	37.5±5.5	0.625
Body mass index (kg/m ²)	25.6±5.3	25.3±4.6	0.588
Parity (n)	3.4±2.1	3.5±2.6	0.708
Age of menarche (years)	13.4±1.3	13.2±1.6	0.123
Duration of menses (days)	6.0±1.6	5.9±1.6	0.817
Menstrual cycle (days)	28.6±2.2	28.5±2.9	0.686
Bleeding, n (%)	93 (60.0)	9 (4.5)	<0.001
Pain, n (%)	43 (28.0)	13 (7.0)	<0.001

Unless otherwise stated, values are presented as means ± standard deviation. UL, uterine leiomyoma.

The digested products were electrophoresed on 3% agarose gel at 90 V for 80 min and visualized by ethidium bromide staining.

In silico analysis. DNA sequences of *CCND1* gene were obtained from The National Center for Biotechnology Information (accession no. CR536538.1). The coding sequence domain of CCND1 was translated using the ExPASy server (<http://web.expasy.org/translate/>). Mode 2 of the RNAsnp (<http://rth.dk/resources/rnasnp/>) was used to evaluate the effects of G870A transition on the CCND1-mRNA structure. RNAsnp is a server that predicts the effect of single nucleotide polymorphisms (SNPs) on the local RNA secondary structure based on the RNA folding algorithms (15). In the graphic summary of the RNAsnp report, the local region, which detects maximum structural change, is colored according to the P-value. If P>0.2 the region is colored black, indicating that the structural change that occurred is not significant.

Statistical analysis. All statistical analyses were conducted using SPSS version 20 (IBM SPSS, Armonk, NY, USA). Demographic characteristics of the groups were analyzed through independent Student's t-test or Fisher's exact test. Logistic regression analysis assessed the independent effect of each polymorphism risk on UL. Odds ratios (ORs) and 95% confidence intervals (CIs) were considered for significant allelic and genotypic associations. Using the χ^2 test, genotype frequencies of the polymorphism were evaluated for deviation from Hardy-Weinberg equilibrium. P<0.05 was considered to indicate statistically significant differences.

Results

Study characteristics. The demographic characteristics of the women with UL and the control group subjects are provided in Table I. The age, BMI and parity (total number of births after 20 weeks) were not significantly different between the UL women and control subjects. No significant differences in menstrual histories, including age of menarche, menstrual

Table II. Comparison of the genotypic and allelic frequency of the cyclin D1 (rs9344) polymorphism in UL women and the control group.

Polymorphism	Control subjects (n=197)	UL women (n=154)	P-value	OR (95% CI)	P-value ^a	OR (95% CI) ^a
Cyclin D1 (rs9344)						
GG, n (%)	50 (25.4)	31 (20)	-	1	-	1
GA, n (%)	105 (53.3)	70 (45.5)	0.79	0.9 (0.5-1.6)	0.89	1 (0.6-1.7)
AA, n (%)	42 (21.3)	53 (34.5)	0.02	1.4 (1.1-1.9)	0.02	1.4 (1.1-2)
Allele						
G, n (%)	205 (52)	132 (43)	-	1	-	-
A, n (%)	189 (48)	176 (57)	0.02	1.5 (1.1-2)	-	-

^aAdjusted for age, body mass index and ethnicity. UL, uterine leiomyoma; OR, odds ratio; CI, confidence interval.

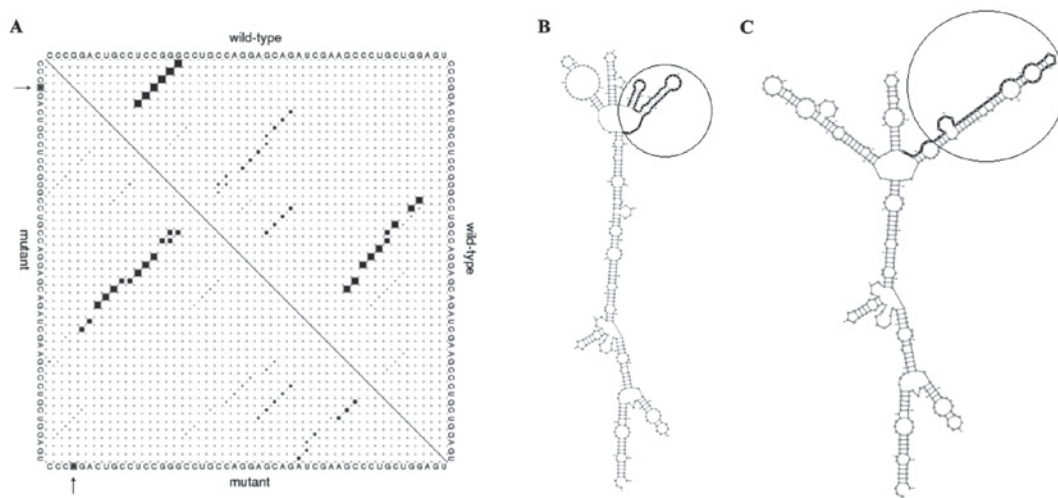


Figure 1. G870A transition effects on CCND1-mRNA were analyzed by RNAsnp. (A) Local region with maximum differences in wild-type and mutant CCND1-mRNA. Base pair probabilities of the local region (523-888) detected with the maximum differences depicted. The upper and lower triangle of the matrix represents the base pair probabilities of wild-type and mutant sequences, respectively. The mutated nucleotide is presented by the arrow. (B) The optimal secondary structure of global wild-type sequence (523-888; circled) with a minimum free energy of -138.70 kcal/mol. (C) The optimal secondary structure of global mutant sequence (523-888; circled) with a minimum free energy of -134.50 kcal/mol.

cycle and duration of menses were identified among the women with UL and the control subjects. The frequency of bleeding and pain was observed to be more frequent in women with UL ($P < 0.0001$).

Genotypes and UL risk. The allelic and genotypic frequencies of the CCND1 G870A polymorphism in women with UL and the control subjects are presented in Table II. The CCND1 G870A polymorphism conformed with Hardy-Weinberg equilibrium ($P > 0.05$). The frequency of GG, GA, and AA genotypes were 20, 45.5 and 34.5%, respectively in the women with UL, and 25.4, 53.3 and 21.3%, respectively in the control group. The frequency of the AA genotype was significantly higher in women with UL compared with the control subjects, although the frequency of the GA genotype was not statistically different between the two groups before and after adjusting for age, BMI, and ethnicity (dominant model); thus, the risk of UL was 1.4-fold greater in women with the AA genotype when compared to the GG genotype before and after adjusting

for age, BMI, and ethnicity (OR, 1.4; 95% CI, 1.1-2; $P = 0.02$). Furthermore, the frequency of the CCND1 870A allele was significantly higher in the women with UL when compared with the control subjects (57 vs. 48%; $P = 0.02$).

Structural analysis. *In silico* analysis indicated that rs9344 (G>A) polymorphism is located at positions 950, 723 and 241 of the transcript, CDS and CCND1 protein, respectively, which alters the CCG codon to CCA. Since these two codons translated to proline, this mutation does not change the protein features of CCND1, including molecular weight, isoelectric point and physicochemical properties (data not shown). Predicting 870AA effects on the local RNA secondary structure of CCND1 revealed that the SNPs cause fundamental changes to the secondary structure of mRNA (distance, 0.1922; $P = 0.1488$; $P < 0.2$ indicates significant structural change) (Fig. 1). The base pair probabilities of the local region detected with maximum differences locate between base pairs 523 and 888 (Fig. 1A). Furthermore, the optimal

secondary structure of global wild-type and mutant sequences are presented in Fig. 1B and C.

Discussion

In the current study, a higher frequency of the *CCND1* 870AA genotype was identified in women with UL when compared to the GG genotype, thus, *CCND1* 870AA homozygous polymorphism may increase the risk of UL.

CCND1 is an important protein in regulation of the cell cycle, causing the transition from the G₁ to the S phase of the cell cycle. *CCND1* regulates cell cycle progression by activating CDK4 and CDK6 (7,8) and the activated enzymes phosphorylate the retinoblastoma (Rb) protein. The phosphorylation of Rb releases the transcriptional factor, E2F, which subsequently triggers numerous downstream genes that are necessary for the cell cycle (16).

Altered expression levels of cell cycle regulatory proteins, such as *CCND1*, are associated with cell proliferation and are present in different types of human tumor and cancer, including endometrial (9,17) and cervical (18) cancer, and endometriosis and adenomyosis (19). An elevated expression level of the *CCND1* protein was observed in leiomyomas compared with the adjacent myometrium during all menstrual cycle phases in a study by Kovács *et al* (20). There are several genetic variants in cell cycle regulatory proteins, which affect protein expression and prevent or stimulate cell proliferation, increasing the susceptibility to tumor development. Hence, molecular epidemiologic studies are considered to be useful strategies for investigating the association between genetic variations and tumorigenesis (21).

In 1995, Betticher *et al* (13) described a G870 to A SNP at codon 242 on exon4 in the *CCND1* gene, which affected splicing of the *CCND1* transcript. *CCND1* mRNA exhibits different splicing, in which translation of the transcripts produces various proteins with dissimilar c-terminal domains. The *CCND1* G870A polymorphism may control splicing of the transcripts, and the 870A allele generates the truncated transcript, encoding a protein with a longer half-life; however, the G870 allele produces the full transcript (13). Therefore, different studies have considered the *CCND1* 870A allele as a susceptible variant in tumorigenesis. Although, there are numerous reports on the correlation between the *CCND1* G870A polymorphism and different types of cancer (22,23), to the best of our knowledge, there is only one published study regarding the association between the *CCND1* G870A polymorphism and UL susceptibility in obese Korean women (14). In 2008, Han *et al* (14) demonstrated that the AA genotype and A allele of the *CCND1* G870A polymorphism may increase UL risk in Korean women with a BMI >25 kg/m² (2), confirming the results of the current study.

Although G870A transition does not change amino acid substitution in the *CCND1* protein, this SNP may alter the *CCND1*-mRNA structure and function. The mRNAs containing different nucleotides at an SNP position may differ in their interactions with cellular molecules involved in mRNA transport, maturation, translation or degradation (24). Analysis of the effects of G870A transition on the secondary structure of mRNA indicated that this SNP may alter the structure and minimum free energy of mRNA. Therefore, G870A may alter

CCND1 gene expression levels. Although the current results of *in silico* analysis improve our understanding of the effects of G870A on the *CCND1* mRNA, further studies are required to evaluate the influence of this SNP on UL susceptibility.

There were certain limitations of the current study. As the conclusion is based on a small number of patients and control subjects, the statistical power is not considered to be particularly high. In addition, the study would be more valuable if myomatous and intact adjacent tissue samples had been investigated. Finally, as there are different ethnic groups in Iran, further studies are required with greater sample sizes from each ethnic group to confirm or refute our findings.

In conclusion, the findings of the present study revealed that the *CCND1* 870AA genotype and *CCND1* 870A allele were associated with a predisposition for UL, and that this polymorphism may be a risk factor for UL. Further studies with greater sample sizes from different populations are required to validate the current findings.

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