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# Cyclin D1 G870A Polymorphism is Associated with an Increased Risk of Simple Endometrial Hyperplasia in Egyptian Women

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## Abstract

Endometrial hyperplasia (EH) is a common diagnosis in women presenting with abnormal uterine bleeding, leading to cancer if untreated. Cyclin D1 A870G polymorphism was associated with increased risk of endometrial carcinoma, but no reported study has evaluated the association between the cyclin D1 A870G polymorphism and the risk of EH. We aimed to study the association of cyclin D1 A870G polymorphism with the risk of simple endometrial hyperplasia (SEH) in Egyptian women. Results showed that A allele was associated with a significantly elevated OR of 3.2 (95% CI =2.15 -5.01, P = 0.0001) in SEH cases, and was found to be associated with a significantly elevated OR of 4.03 (95% CI = 2.3 -7.1, P = 0.000) in premenopausal cases, as well as postmenopausal cases (OR of 2.3, 95% CI = 1.18-4.4, P = 0.01). Using the GG genotype as the reference genotype, the AA genotype was associated with a significantly elevated OR of 8.9 (95% CI = 3.7-21.5, P = 0.0001) in SEH cases, and was found to be associated with a significantly elevated OR of 16.4 (95% CI = 4.8-55.5, P = 0.000) in premenopausal cases as well as post-menopausal cases (OR of 4.6 (95% CI = 1.18-18.1, P = 0.02). In conclusion, the common G to A polymorphism in the CCND1 gene is associated with an increased risk of simple endometrial hyperplasia.

**Keywords:** Cell cycle; Cyclin D1; Endometrial hyperplasia.

## Introduction

Endometrial hyperplasia (EH) is a common diagnosis in women presenting with abnormal uterine bleeding, and can progress to cancer if left untreated [1]. It is characterized by an increased amount of glandular tissue compared to stroma, with architectural features that sometimes include atypical cytologic changes [2]. The risk factors for the occurrence of EH are related to an imbalance of excess estrogen as compared to progesterone, resulting in stimulation of endometrial cell growth [3,4].

Genetic basis for development of EH have been proposed: genetic polymorphism of cytochrome P4501A1 (CYP1A1) [5], phosphatase and tensin homolog gene (PTEN) [6], and matrix metalloproteinase-1 were found to associate with endometrial hyperplasia [7].

The Cyclin-D1 gene (CCND1) is located on 11q13 chromosome and plays a role of a cellular oncogene. The amplification or over expression of the CCND1 gene is common in a variety of different cancers where it induces proliferation [8].

Cyclin D1 plays an important role in the transition from the mid-G1 phase to the S phase of the cell cycle [9]. It is a key sensor and integrator of extra-cellular signals, such as growth factors and hormones. It modulates local chromatin structure of genes involved in regulation of cell proliferation and differentiation. Moreover, cyclin D1 forms physical associations with several transcription factors or transcriptional co-regulators [10,11].

The relationship between Cyclin D1 A870G polymorphism was associated with increased risk of endometrial carcinoma as reported by Kang et al. [8]. Until now, this gene has not been studied in cases of EH, and it remains to be answered whether cases of EH are associated with cyclin D1 polymorphism or not.

The aim of this work was to study the cyclin D1 A870G polymorphism as the impact of multiple complications of simple endometrial hyperplasia (SEH) in Egyptian patients.

## Subjects and Methods

### Study population

The study group included 80 patients with simple endometrial hyperplasia were recruited from the outpatient clinic of Obstetrics and Gynecology Department, Faculty of Medicine, Mansoura University, Egypt during the period from January 2011 and August 2013. Their age ranged from 40-60 years with the mean age of 48.40 ± 4.30 patients with uterine fibroid, associated cancers of endometrium, ovary, and colon were excluded, as well as women who received any hormonal therapy during the last 3 months prior to the study.

The control group included 112 healthy women with comparable age, were selected early in the follicular phase of menstrual cycle. All cases were subjected to history taking for age at menarche, parity, prior use of combined oral contraception (COC): body mass index (BMI) was calculated. All cases give an informed consent; the study protocol was approved by Mansoura ethical committee.

### DNA extraction

A 5 mL sample of venous blood was collected from each subject into a test tube containing EDTA as anticoagulant. DNA was extracted from whole blood using fermentas DNA Purification Kit, based on

|  | SEH cases n=80 | Controls n= 118 | p |
|---|---|---|---|
| Age (y) | 48.40±4.30 | 49.5±5.70 | 0.60 |
| Age at menarche (y) | 13.50± 5.30 | 12.90± 3.40 | 0.40 |
| Prior use of COC<br>Yes (%)<br>No (%) | 56 (70 %)<br>24 (30 %) | 80 (68 %)<br>38 (32 %) | 0.32<br>0.20 |
| Parity Range Median | 0- 43 | 0- 6 4 | 0.35 |
| BMI (kg/m$^2$) | 27.23 ± 5.30 | 26.21 ± 4.90 | 0.46 |
| Endometrial thickness (ET) mm | 14.7 ± 3.90 | 8.5 ± 4.50 | >0.05 |

**Table 1:** Demographic characteristicsand selected endometrial hyperplasia risk factors among the patients and controls.

that blood samples are digested with proteinase K in the supplied lysis solution. The lysate was then mixed with ethanol and loaded onto the purification column, where DNA bound to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the elution buffer.

### Polymerase chain reaction

Polymerase chain reaction (PCR) analysis was performed to determine the genotype of the G/A polymorphism (rs603965) of CCND1 gene at nucleotide 870. Genotyping for CCND1 A870G polymorphisms was done using PCR-RFLP method. The primers for analysis were 5'-GTGAAGTTCATTTC-CAATCCGC-3' and 5'-GGGACATCACCCTCACTTAC-3'. The PCR was done in a Biometra T Gradient Thermocycler. Each 25 AL of PCR mixture contained 10 ng DNA, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0)], 1.5 mmol/L MgCl$_2$ , 0.16 mmol/L each of deoxynucleotide triphosphate, 0.4 μmol/L of each primer, and 1 unit of Taq DNA polymerase. The reaction mixture was initially denatured at 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. The PCR was completed by a final extension cycle at 72°C for 7 minutes. Each PCR product (10 AL) was digested with 15 units of NciI (New England BioLabs, Beverly, MA) at 37°C for 3 hours.

The DNA fragments are then separated and visualized by electrophoresis on 3% agarose gel containing ethidium bromide. The A → G substitution at nucleotide 870 in exon 4 creates an NciI cleavage site. The PCR product (167 bp) with the G allele was digested to two fragments (145 and 22 bp), whereas the PCR product with the A allele was not cut by NciI [12]. The genotyping results were confirmed by a second laboratory research assistant and 5% of the samples were re-genotyped with 100% concordance. Any sample where a genotype could not be accurately assessed was re-genotyped, and the overall call rates were in the range from 99.7–100%.

### Statistical analysis

Polymorphisms and genotype frequencies were evaluated by gene counts. The data were tested for the goodness of fit between the observed and expected genotype frequencies (X2 test). When the observed genotype frequencies fit to Hardy-Weinberg equilibrium, $X_2$ tests (2-by-2 tables; Graphpad Instat Package, Graphpad Software Inc.) were performed to calculate significantly different genotype distributions between patients and controls.

### Results

In (Table 1), there was no significant difference regarding age, age at menarche, prior use of COC, parity, and BMI, between both groups; however there was a statistically significant increase in the endometrial thickness in SEH cases compared to the control (p < 0.05).

Using G allele as reference, the A allele was associated with a significantly elevated OR of 3.2 (95% CI = 2.15 -5.01, P = 0.0001) in SEH cases. Using the GG genotype as the reference genotype, not only the AA genotype was associated with a significantly elevated OR of 8.9 (95% CI = 3.7-21.5, P = 0.0001), but also dominant genotypes (AA plus GA) were associated with a significant increase in SEH cases compared to controls (OR = 0.28 95% CI = 0.13-0.6, p=0.001).

Moreover, using recessive genotypes (GG plus GA) as a reference, there was a significant increase in AA genotype in SEH cases compared with control (OR of 5.9, 95% CI = 3.02-11.6, p=0.0001) as shown in Table 2.

SEH cases included 45 patients in premenopausal age and 35 patients in postmenopausal age. On the other hand, control group included 75 patients in premenopausal age and 43 patients in postmenopausal age. The A allele was found to be associated with a significantly elevated OR of 4.03 (95% CI = 2.3 -7.1, P = 0.0001) in premenopausal cases, as well as postmenopausal cases (OR of 2.3, 95% CI = 1.18-4.4, P = 0.01).

In pre-menopausal cases, using the GG genotype as the reference genotype, the AA genotype was associated with a significantly elevated OR of 16.4 (95% CI = 4.8-55.5, P = 0.0001) in SEH cases compared to control groups. Also dominant genotypes (AA plus GA) were significantly increased in SEH cases compared to controls (OR = 0.2, 95% CI = 0.1-0.7, P = 0.005). Using recessive genotypes (GA plus and GG) as the reference genotype, AA genotype was associated with a significantly elevated OR of 12.02 (95% CI = 4.3-33.2, P = 0.0001) as shown in Table 3.

In post-menopausal cases, using the GG genotype as the reference genotype, the AA genotype was associated with a significantly elevated OR of 4.6 (95% CI = 1.18-18.1, P = 0.02) in cases of SEH.

### Discussion

We studied the frequency distributions of the different genotypes for the CCND1 polymorphism (A870G) in SEH cases and controls.A allele was more frequent among SEH case compared to controls (Tables 2 and 3). We found a statistically significant association between the AA genotype and increased risk in SEH (OR= 8.9, 95% CI = 3.7-21.5, P = 0.000). Also, the AA genotype was associated with elevated OR of 5.9 (95% CI = 3.02-11.6, p=0.000) when using the recessive genotypes (GG plus GA) as a reference genotypes.Moreover, the dominant genotypes (GA plus AA) was significantly increased in SEH case compared to controls (OR = 0.28, 95% CI = 0.13-0.6, p = 0.001) (Table 2).

According to the best of our knowledge, this is the first report for the association between cyclin D1 (A870G) polymorphism and SEH susceptibility. The presence of the A allele has been reported to be positively associated with increased risk for several cancers [10,13,14].

The AA genotype of CCD1 was associated with an increased risk of

|  | SEH 80 case No (%) | Control 118case No (%) | p | OR (95% CI) |
|---|---|---|---|---|
| Allele frequency G A | 51(31.88%) 109(68.13%) | 143(60.59%) 93(39.41%) | 0.0001 | 1.00 (Reference) 3.2(2.15 -5.01) |
| General genotype GG GA AA | 11(13.75%) 29(36.25%) 40(50%) | 42(35.6%) 59(50%) 17(14.4%) | 0.2 0.0001 | 1.00 (Reference) 0.5(0.2-1.18) 8.9(3.7-21.5) |
| Dominant genotype GG AA+ GA | 11(13.75%) 69(86.25%) | 42(35.6%) 76(64.4%) | 0.001 | 1.00 (Reference) 0.28(0.13-0.6) |
| Recessive genotype GG+ GA AA | 40(50%) 40(50%) | 101(85.6%) 17(14.4%) | 0.000 | 1.00 (Reference) 5.9(3.02-11.6) |

**Table 2:** Association between the Cyclin D1 G870A genotype and risk of simple endometrial hyperplasia.

|  | Premenopausal | | | | Postmenopausal | | | |
|---|---|---|---|---|---|---|---|---|
|  | SEH cases n (%) | Control n (%) | P | OR (95% CI) | SEH cases n (%) | Controls n (%) | P | OR (95% CI) |
| Allele frequency G A | 29(32.22%) 61(67.78%) | 99(66%) 51(34%) | 0.0001 | 1.00 (Ref) 4.03(2.3 -7.1) | 22(31.43%) 48(68.75%) | 44 (51.16%) 42(48.84%) | 0.01 | 1.00 (Ref) 2.3(1.18-4.40) |
| General geno-type GG GA AA | 7 (15.60%) 15 (33.30%) 23 (51.10%) | 30(40%) 39(52%) 6(8%) | 0.3 0.0001 | 1.00 (Ref) 0.60(0.2-1.6) 16.40(4.8-55.5) | 4(11.40%) 14(40%) 17(48.60%) | 12(27.90%) 20(46.50%) 11(25.60%) | 0.26 0.02 | 1.00 (Ref) 0.47(0.12-1.7) 4.6(1.18-18.1) |
| Dominant geno-type GG AA+ GA | 7 (15.60%) 38 (84.40%) | 30(40%) 45(60%) | 0.005 | 1.00 (Ref) 0.20(0.1-0.7) | 4(11.40%) 31(88.60%) | 12(27.90%) 31(72.10%) | 0.07 | 1.00 (Ref) 0.33(0.09-1.14) |
| Recessive genotype GG+ GA AA | 22 (48.90%) 23 (51.10%) | 69(92%) 6(8%) | 0.0001 | 1.00 (Ref) 12.02(4.3-33.2) | 18(51.40%) 17(48.60%) | 32(74.40%) 11(25.60%) | 0.09 | 1.00 (Ref) 2.15(0.86-5.30) |

**Table 3** Association between the Cyclin D1 G870A genotypes and endometrial hyperplasia risk in Premenopausal and Postmenopausal of SEH patients and controls.

endometrial carcinoma [8]. Also, Qudduset al. and Balan et al. found an overexpression of CCND1 increases from normal endometrium to endometrial hyperplasia and carcinoma [15,16]. Cyclin D1 exhibits many characteristics of cellular oncogenes [17,18].

The possible mechanisms by which the AA genotype of CCDN1 increase the risk of SEH may be related to one or more of the following factors: (1) CCND1 gene modulates the transcription of CCND1 protein, [19-21]. It has been reported that CCND1 mRNA is alternatively spliced to produce two transcripts (a and b), which are present simultaneously in a variety of normal tissues and cancer cells. The CCND1 protein has 2 subtypes (a and b), they differ in the C-terminal PEST (proline-serine-threonine) -rich region (destruction box) encoded by exon 5 which is responsible for rapid intracellular degradation and turnover of the G1 cyclins, the sub-type b lacks this area, and shows a greater resistance to degradation. [22,23]. The A allele is a major source of variant transcript b in several types of cancer cells [24]. The AA genotype increases the products of transcript b in tumor tissue cells, resulting in an increase of an altered protein with increased half-life [22,25]. Moreover, subtype a of CCDN1 is a better catalyst of RB (retinoblastoma protein) phosphorylation/inactivation [24,26]. (2) Also, CCDN1 can activate CDK4or CDK6 to phosphosphorylate a series of key substrates, such as RB, in that case, the transcription factors will be released to promote synthesis of DNA and accelerate thecells proliferation [27], (3) It is a key sensor and integrator of extra-cellular signals, such as growth factors and hormones [11]. (4) It modulates local chromatin structure of genes involved in regulation of cell proliferation and differentiation through binding both the cyclin-dependent kinases and histone acetylase and deacetylases[23]. (5) It forms physical associations with several transcription factors or transcriptional co-regulators [10,11]. (6) The activation of CCDN1 stimulates the transcription of genes regulated by estrogen receptors (ER) and progesterone receptors (PR) [16].

Experimental over-expression of D-type cyclins in cell lines shortens the duration of G1, reduces the requirement for exogenous growth factors, and can prevent terminal differentiation [28,29].

Also, in the present study, among premenopausal cases of EH we found the AA genotype was associated with a significantly elevated OR of 16.4 (95% CI = 4.8-55.5, P = 0.0001). The dominant genotypes (AA plus GA) was associated with increased risk of EH (OR=0.2, 95% CI = 0.1-0.7, P = 0.005). In addition, AA genotype was associated with a significantly elevated OR of 12.02 (95% CI = 4.3-33.2, P = 0.0001) versus recessive genotypes (GG plus GA). In postmenopausal cases the AA genotype was associated with increased risk of EH (OR of 4.6, 95% CI = 1.18-18.1, P = 0.02). However, either the dominant genotypes (AA plus GA) or the recessive genotypes was not associated with increased risk of EH (P 0.07, 0.06) respectively.

The actual causes for these differences between pre-menopausal and post-menopausal cases were not properly known. It may be related to small number of cases in both groups; also, the present study was a hospital-based case–control study; patients were selected at a single institution. It may indicates a different mechanisms for the

occurrence of SEH between these age groups as estrogen level is lower in postmenopausal than premenopausal cases. Therefore, a further multicenter study including a large number of patients should be done to clarify or refute our findings.

## Relation between CCDN1 and estrogen in simple endometrial hyperplasia

One of the most important risk factors of SEH is excess estrogen as compared to progesterone, resulting in the stimulation of endometrial cell growth [3,4]. Estrogens induces cell proliferation in target tissues by stimulating progression through the G1 phase of the cell cycle, and induction of CCDN1 expression is a critical feature of the mitogenic action of estrogen [30]. In turn, cyclin D1 has been reported to enhance the trans-activating effects of estrogens, independently of its role in the regulation of cyclin-dependent kinase activities [30]. The CCDN1 can bind directly to the estrogen receptor, trans-activate estrogen receptor response elements [31], and regulate estrogen-dependent enhancer activity [32]. Therefore, estrogen exposure and functional genetic polymorphism of the CCDN1 gene may synergistically increase the risk of endometrial hyperplasia as explained by [12].

## Conclusions

In conclusion, this case-control study shows that the common G to A polymorphism in the CCDN1 gene is associated with an increased risk of simple endometrial hyperplasia.

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