Association of *p53* and *CDKN1A* Genotypes with Endometriosis

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Abstract. Background: The tumor suppressor p53 protein plays a critical role in different cellular processes in response to DNA damage and it is responsible for transcriptional induction of the *p21* (CDKN1A/WAF1/CIP1) gene. Both p53 and p21 are thought to play major roles in the development of human malignancy. Polymorphic variants of *p53* at codon 72, and *CDKN1A* at codon 31, have been found to be associated with cancer susceptibility, but few studies have investigated their effect on endometriosis risk. Materials and Methods: In this hospital-based case control study, we investigated the association of *p53* codon 72 and *CDKN1A* codon 31 polymorphisms with endometriosis susceptibility in a Taiwanese population. In total, 180 patients with endometriosis, and 330 age-matched controls in Central Taiwan were recruited and genotyped.

Results: We found a significant difference in the distribution of the *p53* genotype, but not the *CDKN1A* genotype, between the endometriosis and control groups. Individuals with the C (Pro) allele at *p53* codon 72 had a 1.6-fold increased odds ratio of endometriosis, and those with Arg/Pro and Pro/Pro genotypes for *p53* codon 72 had a 1.84- and 2.74-fold (95% confidence interval=1.17-2.92 and 1.58-4.74) increased risk of

endometriosis compared to those with Arg/Arg, respectively. The distribution of haplotype combinations of *p53* codon 72 and *CDKN1A* codon 31 was statistically different in the endometriosis and control groups. The percentages of the three subgroups with *p53* CC homozygote were all higher in the endometriosis group than in the control group.

Conclusion: Our findings suggest that the C (Pro) allele of *p53* codon 72 may be associated with the development of endometriosis, and could serve as a potential biomarker for early prediction.

Endometriosis is a chronic gynecological disease characterized by growth of endometrial tissue in sites other than the uterine cavity, most commonly in the pelvic cavity, including the ovaries, the uterosacral ligaments, and pouch of Douglas (1). Endometriosis possesses many features of a benign neoplastic process with the potential for malignant transformation (2). Although the overall mechanisms and even the exact prevalence are unknown, several factors are thought to be involved in the development of endometriosis. Generally speaking, retrograde menstruation remains the dominant theory for the development of pelvic endometriosis (3). Recently, genetic studies have reported that some specific genotypes were associated with endometriosis in selective populations, such as Brazil (4), Turkey (5), and Taiwan (6, 7); however, the exact genomic and proteomic factors that play a role in endometriosis are not very clear. Similar to carcinogenesis, genomic alterations may represent important events in the development of endometriosis. Tumor suppressor genes are known to play a role in the regulation of cell growth and prevention of carcinogenesis. Thus, altered tumor suppressor genes might be related with the development of endometriosis (8).

The *p53* gene is a tumor suppressor gene whose function is partially mediated by transactivating the cyclin-dependent kinase inhibitor 1A (*CDKN1A*) promoter, to control the cell cycle and prevent tumor formation (9). It was found that premalignant lesions with mutant p53 protein overexpressed p21 (10). p21-immunopositive well-differentiated tumors with *p53* missense mutations probably harbor a p21-dependent differentiation pathway activated through a p53-independent mechanism (11). There is discrepancy about this presentation of *p53* polymorphisms in various tumor types. The *p53* Arg72 homozygote is considered to be a risk factor in the development of cancer (12). In contrast, some investigators demonstrated no association between the different *p53* polymorphisms and individual cancer development (13). Still other studies revealed a higher risk in individuals homozygous for *p53* Pro72 (14, 15). High frequency of *p53* locus deletion was observed in endometriosis specimens (16). p53 protein abnormalities and chromosomal aberrations may be involved in malignant transformation of ovarian endometriosis (17). In contrast, some investigators have demonstrated no expression of p53 in the endometriosis specimens (18-20).

The protein p21 (CDKN1A/WAF1/CIP1), encoded by the *CDKN1A* locus, is a cyclin-dependent kinase inhibitor which play a role in cell cycle regulation. The human *CDKN1A* gene contains three exons of 68, 450, and 1600 bp (21). In normal cells, p21 exists predominantly in quaternary complexes with cyclins, cyclin-dependent kinases (CDKs), and proliferating cell nuclear antigen (PCNA) to inhibit the activity of CDKs and control the G_1 to S phase transition (22). The *CDKN1A* gene has a *p53* transcriptional regulatory motif, and cells lacking functional p53 express very low levels of p21, suggesting that p53 regulates *CDKN1A* expression directly (23). p21 controls the differentiation of normal and transformed cells, and the involvement of p21 in terminal differentiation has been observed in several studies (24, 25). Differential regulation of p21 by p53 and retinoblastoma protein has been reported in cellular response to oxidative stress (26). In addition, several studies suggest a critical role for p21 in apoptosis (27).

In view of the central role of p21 in inducing growth arrest, terminal differentiation, or apoptosis, aberrant *CDKN1A* genomic and proteomic regulation may play a vital role in the pathogenesis of cancer. Alterations in p21 expression have been observed in specific types of human cancer,

including ovarian, uterine, cervix, colorectal, hepatocellular, and head and neck carcinomas (28-30). As is well known, in response to DNA damage, p21 is a key mediator of the G_0-G_1 cell cycle arrest induced by tumor suppressor p53. It has been revealed that p21 also interacted with PCNA to cause both G_1 and G_2 cell cycle arrest in p53-deficient cells (24, 31, 32). Recently, a novel polymorphism of *CDKN1A* gene in codon 149 was found in an Indian population and was considered as a genetic susceptible marker of esophageal and oral cancer (10, 33). Soon after, bioinformatics analysis revealed that the so-called polymorphism of *p21* codon 149 is not a susceptible site (34). Although the *p21* genotype was found not to be associated with endometriosis, the limited population investigated and this being the only literature is not convincing (35). Thus in this study, we wished to investigate the association of combined genotypes of *p53* and *p21* with endometriosis.

Taken together, these effects reflect the complexity of the p53/p21 pathways of cell cycle regulation and differentiation in overall carcinogenesis. Mutations in either *p53* or *CDKN1A* are detected in some tumor cells (9, 36), and polymorphisms of *p53* codon 72 or *CDKN1A* codon 31 were found to be associated with many tumors (37-40).

Since *p53* gene mutations are the most common cancer-related genetic alterations, being found in ~50% of human cancer cases (41), and p53 regulates p21 expression (23), we were interested to check the susceptible site in *p53* and *CDKN1A* gene together in Taiwan endometriosis patients. Thus, the main goal of the study was to check the joint effect of genotypes in *p53* tumor suppression gene codon 72, and *CDKN1A* gene codon 31, with endometriosis in a Taiwan population.

Material and Methods

Study population and sample collection. We recruited 180 individuals diagnosed with endometriosis at the Outpatient Clinics of General Surgery at the Chung-Shan Medical University Hospital, Taichung, Taiwan, Republic of China. All patients voluntarily participated, completed a self-administered questionnaire and provided their peripheral blood. Three hundred and thirty non-endometriosis and non-cancer healthy individuals as controls were selected by matching for age and gender, from people who voluntarily visited the health-screening clinic at the same hospital. A questionnaire administered to the volunteers

included questions on alcohol consumption habit and smoking history and frequency. Self-reported alcohol consumption and smoking habits were evaluated and classified as categorical variables. Information on these factors was obtained more than twice a week for years. Our study was approved by the Institutional Review Board of Chung-Shan Medical University Hospital and written informed consent was obtained from all participants.

Genotyping assays. Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan), and further processed according to a previous published paper (42). Briefly, for *p53* codon 72, the primers 5'-TCCCCCTTGCCGTCCCAA-3' and 5'-CGTGCAAGTCACAGACTT-3' were used, and for *CDKN1A* codon 31, the primers 5'-GTCAGAACCGGCTGGGGATG-3' and 5'-CTCCTCCCAACTCATCCCGG-3' were used. The following cycling conditions were performed: one cycle at 94℃ for 5 min; 35 cycles of 94 ℃ for 20 s, 58℃ for 20 s, and 72℃ for 20 s; and a final extension at 72 ℃ for 10 min. The PCR products were studied after digestion with

*BstU*1 restriction enzyme for *p53* codon 72, and with *Blp*I for *CDKN1A* codon 31, respectively.

Statistical analyses. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, deviation of the genotypic frequencies of *p53* codon 72 and *CDKN1A* codon 31 single nucleotide polymorphisms in the controls from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's χ^2 test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *p53* and *CDKN1A* genotypes between cases and controls. We estimated the endometriosis risk associated with the genotypes as odds ratio (ORs) and 95% confidence intervals (CIs) by using unconditional logistic regression with adjustment for age, smoking, alcohol consumption and betel quid (BQ) chewing habits. Data was recognized as significant when the statistical *p*-value was less than 0.05.

Results

The mean ages of the endometriosis patients and the controls were 31.3 (standard deviation SD=4.12) and 32.2 (SD=4.58) years, respectively.

There were no differences in the age, body mass index (BMI), smoking and alcohol drinking status between the two groups (data not shown). The frequency of the alleles for *p53* codon 72 and *CDKN1A* codon 31 in the endometriosis and control groups is shown in Table I. The Pro allele at *p53* codon 72 was significantly associated with endometriosis risk (p=0.00036, OR=1.60, 95% CI=1.23-2.07). In contrast, neither Arg nor Ser at *CDKN1A* codon 31, was differently distributed between the endometriosis patients and control groups (p>0.05).

The frequency of the genotype of *p53* codon 72 and *CDKN1A* codon 31 polymorphisms in the endometriosis and control groups is shown in Table II. These data indicate that there was an obvious association between carrying the C allele (72Pro) of *p53* and endometriosis risk. As for these significant stratifications, after controlling for age, smoking, and alcohol drinking status, the adjusted OR was still significant (Table II). On the contrary, neither hetero- nor homozygotes of 31Ser of *CDKN1A* seemed to be risky genotypes for endometriosis (p>0.05) (Table II).

Since *p53* and *CDKN1A* may be closely related to each other in a same pathway, the gene–gene interaction was also investigated. The

result of analysis of the haplotype combinations of *p53* codon 72 and *CDKN1A* codon 31 is shown in Table III, and there was a significant difference between endometriosis and control groups ($p<0.00001$). The percentages of three subgroups with p53 GG homozygote were all higher in the endometriosis group (Table III). The results in Table III again show a major role of *p53* codon Arg72 and a minor role of *CDKN1A* codon Ser31 in endometriosis development, which is indicated in the results of Table I.

Discussion

Cell proliferation and death are essential aspects in the understanding of carcinogenesis. Considerable evidence now links the activities of the *p53* gene to regulation of the cell cycle and mutations in this gene are the most common genetic changes known to occur in human cancer (41). In this study, it was found that these homozygous for *p53* codon 72 Pro allele had a 2.74-fold higher risk for endometriosis development (Table II). As for the Arg/Pro heterozygotes, there was also a 1.84-fold increased risk. Our findings conflict with those of other studies in oncology, which

report the Arg allele to be a risky genotype. The Arg allele has been reported to be associated with a 4.69-fold increased risk for bladder cancer (43) and 3.1-fold higher risk for gastric cardia adenocarcinoma (44). In addition, it is reported that the majority (76%) of female papillomavirus-associated cancer patients were Arg homozygous while only 37% of the controls were (12). Like ours, there are also a few studies which reported the Pro allele to be risky. The Pro allele is associated with a 1.37- to 11.29-fold higher risk for lung cancer (45-47), a 3.7-fold higher risk for nasopharyngeal carcinoma (48), and an 11-year earlier age of onset for oral cancer in non-Hispanic white population (49). Most interestingly, our findings are consistent with the literature investigating a smaller Taiwanese population, of 148 endometriosis patients and 150 health controls (50). In that report, codon 11 and 248 were not polymorphic sites, and codon 72 genotype was found to be significantly associated with endometriosis (50). However, although their ORs may have been overestimated with a limited bias controls criticized by Lee et al (51), our study confirm their findings. Furthermore, we extend the effects of *p53* codon 72 genotype on endometriosis by analyzing the combinatorial genotypes of *p53* and *CDKN1A* together, finding that the

distributions of specific haplotypes, such as CC/AA, CC/AC, and CC/CC, were of higher frequency in the endometriosis group than in the control group (Table III). Our findings and those of Hsieh et al's were different to the negative findings for Japanese (52) and Brazilian populations (53). The discrepancy may be due to the investigating populations being collected from different endometriosis staging, and more likely, to the variations between the two ethnicities.

The p53Arg72 and p53Pro72 proteins do not differ in their ability of binding to DNA in a sequence-specific manner but do differ in other ways. The p53Arg72 protein induces apoptosis faster and suppresses transformation more efficiently than does the p53Pro72 protein (54). Conversely, the p53Arg72 protein is more susceptible to degradation by HPV E6 proteins, and this degradation is correlated with increased risk of HPV-associated cancer (12). However, there is no literature to our knowledge investigating the phenotypic characteristics in endometriosis patients. p21 protein inhibits two different targets, the cyclin-CDK complexes and PCNA, which control cell cycle transitions and DNA replication. Two particularly conserved regions in the human *CDKN1A* gene were found on 60 amino acids near codon 21 and 164 amino acids

near codon 130 (55). Our experimental result shows that there was no significance at codon 31 of *CDKN1A* in the Taiwanese population we have investigated, and it is not a susceptible marker for endometriosis (Table II). These data do not exclude the possibility that *CDKN1A* may play a role in the development of endometriosis, neither can all the other mediators of p53 function, such as p27 and alpha-catenin, be ignored in further study.

In conclusion, this study shows that *p53* codon 72 polymorphism may be involved in the development of endometriosis.

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Table I. Allelic frequencies for *p53* codon 72 and *CDKN1A* codon 31 polymorphisms in the endometriosis and control groups.

Allele	Cases (%)	Controls (%)	OR (95% CI)	*p*-Value[a]
p53 codon 72				
Allele G (Arg)	167 (46.4)	383 (58.3)	1.00 (ref)	
Allele C (Pro)	193 (53.6)	277 (42.0)	1.60 (1.23-2.07)	0.00036
CDKN1A codon 31				
Allele A (Arg)	171 (47.5)	334 (50.6)	1.00 (ref)	
Allele C (Ser)	189 (52.5)	326 (49.4)	1.13 (0.88-1.46)	0.3431

OR, Odds ratio; CI, confidence interval. [a]Based on χ^2 test.

Table II. Association of *p53* codon 72 and *CDKN1A* codon 31 polymorphisms with endometriosis risk.

Genotype	Cases (%)	Controls (%)	Crude OR (95% CI)	Adjusted OR (95% CI)[a]
p53				
Arg/Arg	34 (18.9)	107 (32.4)	1.00 (ref)	1.00 (ref)
Arg/Pro	99 (55.0)	169 (51.2)	1.84 (1.17-2.92)[b]	1.79 (1.15-2.84)[b]
Pro/Pro	47 (26.1)	54 (16.4)	2.74 (1.58-4.74)[b]	2.76 (1.54-5.03)[b]
With Pro	146 (81.1)	223 (67.6)	2.06 (1.33-3.20)[b]	2.03 (1.29-3.23)[b]
With Arg	133 (73.9)	276 (83.6)	1.00 (ref)	1.00 (ref)
Pro/Pro	47 (26.1)	54 (16.4)	1.80 (1.16-2.81)[b]	1.78 (1.14-2.83)[b]
CDKN1A				
Arg/Arg	35 (19.4)	69 (20.9)	1.00 (ref)	1.00 (ref)
Aer/Ser	101 (56.1)	196 (59.4)	1.01 (0.63-1.63)	0.98 (0.64-1.70)
Ser/Ser	44 (24.4)	65 (19.7)	1.34 (0.76-2.33)	1.37 (0.69-2.35)
With Ser	145 (80.6)	261 (79.1)	1.10 (0.70-1.73)	1.01 (0.69-1.86)
With Arg	136 (75.6)	265 (80.3)	1.00 (ref)	1.00 (ref)
Ser/Ser	44 (24.4)	65 (19.7)	1.32 (0.85-2.04)	1.37 (0.79-2.07)

OR, Odds ratio; CI, confidence interval. [a]Adjusted for age and habits (smoking and alcohol drinking habits). [b]$p < 0.05$.

Table III. Distribution of haplotype combinations of *p53* codon 72 and *CDKN1A* codon 31 polymorphisms in the endometriosis and control groups.

Genotype	Cases (%)	Controls (%)	Crude OR (95% CI)	*p*-Value[a]
P53/CDKN1A				
GG/AA	7 (3.9)	22 (6.7)	1.00 (ref)	
GG/AC	19 (10.6)	64 (19.4)	1.07 (0.40-2.89)	
GG/CC	8 (4.4)	21 (6.4)	0.84 (0.26-2.71)	
GC/AA	19 (10.6)	36 (10.9)	0.60 (0.22-1.67)	
GC/AC	56 (31.1)	100 (30.3)	0.57 (0.23-1.41)	
GC/CC	24 (13.3)	33 (10.0)	0.44 (0.16-1.19)	
CC/AA	9 (5.0)	11 (3.3)	0.39 (0.11-1.32)	

CC/AC	26 (14.4)	32 (9.7)	0.39 (0.14-1.06)	
CC/CC	12 (6.7)	11 (3.3)	0.29 (0.09-0.95)[b]	<0.00001[c]

[a]Based on chi-square test. [b]Significant difference. [c]Chi square=172.6228, degree of freedom=8.