MOLECULAR REPRODUCTION AND DEVELOPMENT 74:815–820 (2007)

# Four Novel Single Nucleotide Polymorphisms Within the Promoter Region of p53 Gene and Their Associations With Uterine Leiomyoma

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**ABSTRACT** Dysregulated p53 expression has been implicated as a major contributor to numerous tumorigenesis. Single nucleotide polymorphisms (SNPs) within the functional consequence of the novel p53 promoter region remains obscure. Herein, we aimed to establish the extent of genetic variability within the promoter region of p53 gene as well as their association with leiomyoma susceptibility. Women were divided into two groups, leiomyoma (n = 160) and nonleiomyoma controls (n = 200). Total DNA was isolated from the peripheral blood of subjects. The DNA fragment containing p53 promoter regions (+64 ~ −404 bp) were obtained by amplification of polymerase chain reaction. The variations of DNA fragments were detected by DNA sequencing or restriction fragment length polymorphism (RFLP). Sequence alignment was used to identify sequence variations in p53 promoter regions. Genotypes were analyzed by method of RFLP. Genotypes/allelic frequencies in the leiomyoma and control groups were compared. A total of 15 sequence variations within p53 promoter region were identified, including −408 T/C, −382 A/G, −359 A/G, −325 T/C, −250 A/G, −216 T/C, −205 G/A, −198 G/A, −177 T/C, −103 A/G, −81 G/A, −71 G/A, −51 T/A, −33 A/G, and −17 T/C. Among these variations, four SNPs (−250 A/G, −216 T/C, −103 A/G, and −33 A/G) were established. Allele frequencies of −250*G/−216*C/−103*G/−33*G in the leiomyoma group and control group 6.9/5.0/5.9/3.8% and 3.8/1.8/2.3/4.0%, respectively. Two of them (−216*C and −103*G) are associated with higher leiomyoma susceptibility. We concluded that some sequence variations were observed within the promoter region of p53 gene. The SNPs of −216*C and −103*G among the identical sequence variations are associated with leiomyoma development. *Mol. Reprod. Dev. 74: 815–820, 2007.* © 2006 Wiley-Liss, Inc.

**Key Words:** leiomyoma; p53; p53 promoter; polymorphism; SNP

## INTRODUCTION

Leiomyoma, the most common benign uterine neoplasma, is occurred in around one half of the women during their lifetimes (Cramer, 1972). The myoma growth may be derived from growth and proliferation of a single smooth muscle cell (Townsend et al., 1970). Leiomyoma is related with the complex mechanism of autocrine and paracrine interaction or the effects of sex steroid hormone action on cells (Strawn et al., 1995). Leiomyoma is responsive to the ovarian steroids, estrogen and progesterone. However, a mechanistic understanding of the role of these hormones remains to be elucidated.

Loss of tumor suppressor function generally occurs in tumorigenesis. Somatic mutations in p53 gene are the most common genetic alterations found in human malignancies (Oh et al., 2000). The tumor suppressor p53 gene is mutated in around half of all cancers. It is therefore reasonable to assume that naturally occurring polymorphic genetic variants in the p53 promoter region might determine an individual susceptibility to leiomyoma. Genomic instability of p53 plays a role in the development and progression of various tumor types. Recently, the tumor suppressor gene such as p53 influence growth of leiomyoma is being investigated (Strawn et al., 1995). Altered tumor suppressor genes might be related with the development of leiomyoma (Jiang et al., 1996). Induction of apoptosis may be a mechanism of the effect of GnRHa in leiomyoma (Higashijima et al., 1996).

Functional inactivation of tumor suppressor genes during tumor progression has been shown to occur by either coding region mutation or promoter region. The segregation, substitution, or deletion within the promoter consequences might directly or indirectly interfere with the guarantee of p53 as well as the consequent tumorigenesis. Transcriptional inactivation of the promoter region may participate in carcinogenesis

Capsule of abstract: P53 promoter −216*C and −103*G related genotypes/alleles are associated with leiomyoma susceptibility.

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Received 29 November 2005; Accepted 3 January 2006
Published online 21 December 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20464

(Oue et al., 2001). Transcriptional repression by p53 promoter methylation might contribute to tumor progression (Pogribny and James, 2002). Aberrant methylation of CpG islands within promoter regions of the gene is associated with transcriptional inactivation of various tumor suppressor genes in neoplasms (Oue et al., 2001). Therefore, mutations in p53 promoter region might play a partial role in the process of tumorigenesis. Furthermore, the understanding of the detailed characterization of p53 promoter is useful for the elucidation of the underlying regulation of p53 expression.

Reviewing literature, few investigators demonstrated the mutation statuses of p53 promoter as well as its promoter region in leiomyoma individuals. Some investigators have demonstrated the undetectable expression of p53 in the leiomyoma specimens (Vercellini et al., 1994; Horiuchi et al., 1998; Schneider et al., 1998). No literature revealed the association between the leiomyoma and the p53 promoter genes. In this series, we firstly sequenced the p53 promoter region in individuals with leiomyoma. We aimed to detect the novel sequence variations and determine whether mutations in transcription regulatory sequences of p53 gene may result in leiomyoma development. We also tried to elucidate the relationship between polymorphisms of p53 promoter gene and leiomyoma. This report is the first application in this aspect.

## PATIENTS AND METHODS

Premenopausal Taiwanese women with surgically diagnosed leiomyoma and nonleiomyoma were included. All patients were divided into two groups: (1) Leiomyoma (n = 160) and (2) nonleiomyoma groups (n = 200). Leiomyoma statuses were pathologically confirmed after myomectomy or hysterectomy. In clinical practices, women without leiomyoma would not accept the surgical procedure to confirm the nonleiomyoma statuses. Therefore, the nonleiomyoma statuses were confirmed after detail sonography examination. All women had consented to peripheral blood sampling for genotype analyses. All individuals did not accept hormone therapy 1 year before recruitment. The study was approved by the ethical committee and institutional review board of the China Medical University Hospital. All women accepted the peripheral blood sampling for genotype analyses. Genomic DNA was isolated from peripheral blood using Genomaker DNA extractor kit (Blossom, Taiwan). About 50 ng of genomic DNA was mixed with 20 pmole of PCR primers in a total volume of 25 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphate, and 1 unit of Amplitaq DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA).

PCR primers were synthesized according to the published p53 GenBank promoter sequence (accession no. X54156) spanning a 468-basepair fragment. The sequences of the primers were as following: Forward, 5′-GAT ATT ACG GAA AGC CTT C-3′; Reverse, 5′-AGC CCG AAC GCA AAG TGT-3′. The PCR amplification was performed in a programmable thermal cycler GenAmp PCR system 2400 (Perkin Elmer Applied Biosystems). The PCR conditions were set as follows: One cycle at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and final cycle of extension at 72°C for 3 min.

PCR fragments were purified, cloned into pGEM-Teasy vector (Promega, Madison, WI) and then cyclesequenced for sequence variations using the Thermo Sequenase dye terminator kit and reactions were analyzed on the ALF express automated DNA sequencer (Amersham Pharmacia Biotech, Amersham, UK). The 468-bp PCR products of p53 promoter cloned from the population of 160 individuals with leiomyoma were sequenced and aligned for determining sequence variations. The resultant reads are then compared with the wild genome for Homo sapiens.

The identified single nucleotide polymorphisms (SNPs) were further analyzed by the method of restriction fragment length polymorphism (RFLP). The restriction enzymes were purchased from Biolabs (New England Biolabs, Inc, Beverly, MA). The related enzymes and sequences of cutting sites as well as digestion products are listed in Table 2. All of restriction enzymes digestions were performed according commercial instructions. Genotypes and allelic frequencies for p53 promoter polymorphisms in the leiomyoma and control groups were compared. Allelic frequencies are expressed as a percentage of the total number of alleles. The SAS system (Version 8.1, SAS Institute Inc., Cary, North Carolina) with $\chi^2$ test was utilized for statistical analyses. A $P$ value $<0.05$ was considered statistically significant.

## RESULTS

A 468-bp PCR product of p53 promoter cloned from the population of 160 individuals with leiomyoma were sequenced and aligned for determining sequence variations. The resultant reads are then compared with the wild genome for Homo sapiens. There were 15 sequence variations were identified. These mutations were single base substitutions, including −408 T/C, −382 A/G, −359 A/G, −325 T/C, −250 A/G, −216 T/C, −205 G/A, −198 G/A, −177 T/C, −103 A/G, −81 G/A, −71 G/A, −51 T/A, −33 A/G, and −17 T/C (Fig. 1). The percentages of mutated sequences detected are ranging from 1.3 to 6.9% (Table 1). Among these 15 mutated points, the −250 A/G, −216 T/C, −205 G/A, and −33 A/G with percentages ≥5.0% were identified and they were selected for further RFLP assay (Fig. 2).

The SNPs of −250 A/G, −216 T/C, −205 G/A, and −33 A/G were identified by the restriction enzymes of *Bgl* I, *Mae* I, *Tau* I, and *Dde* I digestion, respectively (Table 2). Allele frequencies of −250*G/−216*C/−103*G/−33*G in the leiomyoma group and control group 6.9/5.0/5.9/3.8% and 3.8/1.8/2.3/4.0%, respectively. Among these four SNPs, two of them (−216 T/C and −205 G/A) appeared different distributions between individuals with and without leiomyoma (Table 3). Alleles of −216*C and −103*G within the promoter region of

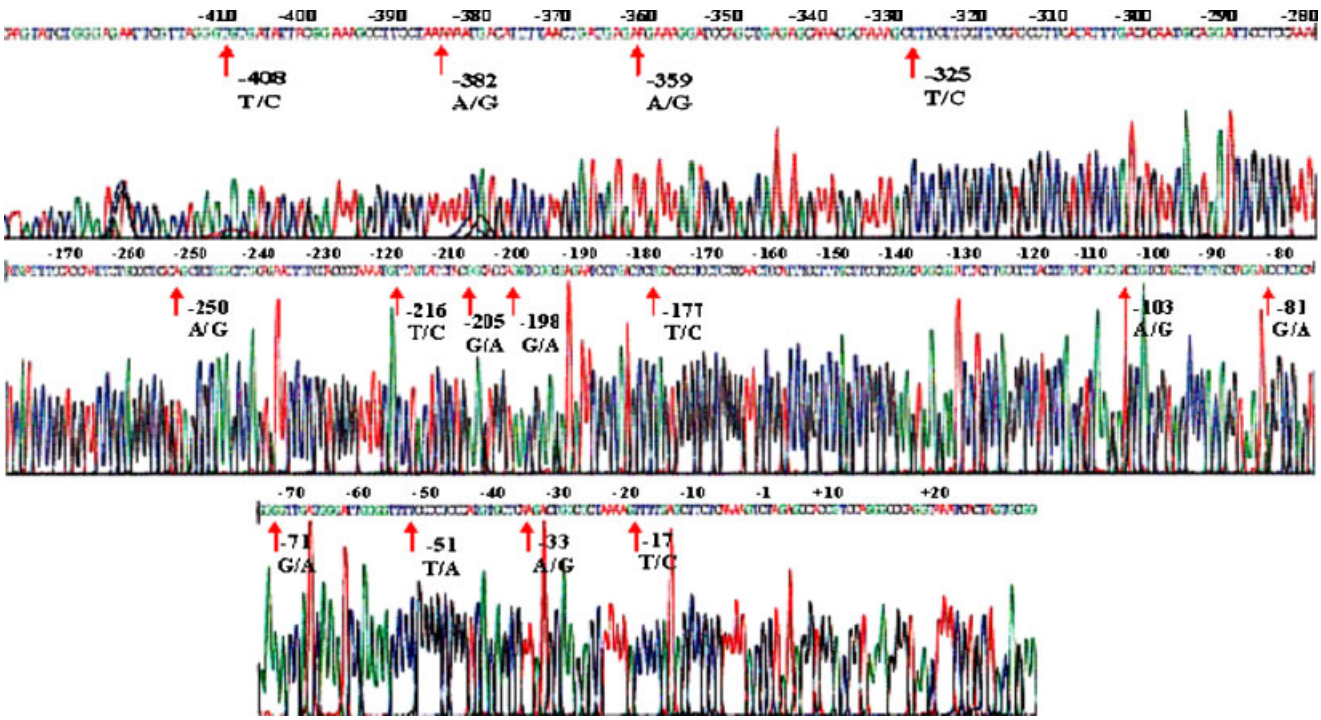


**Fig. 1.** A total of 15 sequence variations identified in the p53 promoter regino. These SNP located at −408 T/C, −382 A/G, −359 A/G, −325 T/C, −250 A/G, −216 T/C, −205 G/A, −198 G/A, −177 T/C, −103 A/G, −81 G/A, −71 G/A, −51 T/A, −33 A/G, and −17 T/C. [See color version online at www.interscience.wiley.com.]

p53 genes were associated with higher susceptibility of leiomyoma development.

## DISCUSSION

Leiomyoma is the most common tumor in women, but its etiology remains unclear. Estrogen may exert its mitogenic effects on leiomyoma through estrogen-dependent growth factors (Friedman et al., 1990). However, sex steroid hormones control uterine growth mechanisms not only via an influence on proliferation but also via apoptosis (Wu et al., 2000). Apoptosis is important for the proper maintenance of homeostasis in a tissue and the removal of damaged or excess cells from population. The balance between cell proliferation and cell death determines tumor growth. Disregulation between proliferative and apoptotic responses may contribute to the disruption of tissue homeostasis and neoplastic growth of leiomyoma (Burroughs et al., 2000).

**TABLE 1. The Percentages of Mutated p53 Promoter Genes in the Population (n = 160) of Leiomyoma Group**

| Position | Wild-type → mutated-type | Case no. (%) of mutated-type |
|---|---|---|
| −408 | T → C | 5 (3.13) |
| −382 | A → G | 2 (1.25) |
| −359 | A → G | 4 (2.50) |
| −325 | T → C | 3 (1.88) |
| −250 | A → G | 11 (6.88)* |
| −216 | T → C | 9 (5.63)* |
| −205 | G → A | 2 (1.25) |
| −198 | G → A | 2 (1.25) |
| −177 | T → C | 5 (3.13) |
| −103 | A → G | 9 (5.63)* |
| −81 | G → A | 7 (4.38) |
| −71 | G → A | 6 (3.75) |
| −51 | T → A | 7 (4.38) |
| −33 | A → G | 8 (5.00)* |
| −17 | T → C | 2 (1.25) |

The sequence was detected by the method of DNA sequencing.
*Identified single nucleotide polymorphism.


**Fig. 2.** Four single nucleotide polymorphisms (−250 A/G, −216 T/C, −103 A/G, and −33 A/G) were identified within the promoter region of p53 gene.

**TABLE 2. The Cutting Sequences, Restriction Enzymes, and DNA Fragments After Digestion for p53 Promoter −250 A/G, −216 T/C, −103 A/G, and −33 A/G Polymorphisms**

| SNPs | Sequence of cutting site | Restriction enzyme | Allele type (no. of cutting site) | DNA fragment (bp) |
|---|---|---|---|---|
| −250 A/G | $GCC(N)_4$^NGGC | *Bgl* I | WT (0) | 468 |
| | | | MT (1) | 153, 315 |
| −216 T/C | C^TAG | *Mae* I | WT (1) | 61, 407 |
| | | | MT (2) | 61, 189, 218 |
| −103 A/G | GCSG^C | *Tau* I | WT (0) | 468 |
| | | | MT (1) | 166, 302 |
| −33 A/G | C^TNAG | *Dde* I | WT (1) | 60, 408 |
| | | | MT (2) | 40, 60, 368 |

N indicates G, A, T, or C.
WT, wild-type; MT, mutant type.

The regulation of p53 expression might be mediated through binding of individual proteins to target sites at p53 promoter regions.

P53 gene and its encoded protein are related with the regulation of cell cycle, cellular growth, and apoptosis. It is a gatekeeper or guardian of cell division (Lane, 1992; Levine, 1997). The p53 mutations are associated with instability of cell development and cycle progression (Harris and Hollstein, 1993). Dysregulated p53 expression has been implicated in the pathogenesis of a number of diseases. Alterations of p53 are related to the induction of apoptosis in malignant tumors. Individuals lacking functional p53 are at an increased risk of tumor development. A mutated p53 gene or malfunctioned p53 protein has often observed in patients with numerous malignancies (Harris and Hollstein, 1993).

There is discrepancy about the distribution of p53 polymorphism in different malignancy. Numerous cancers are related with the abnormal p53 presentation, including cervical carcinoma (Zehbe et al., 1999), ovarian carcinoma (Kupryjanczyk et al., 1994), bladder cancer (Esrig et al., 1994), prostate cancer (Steiner et al., 2000), hepatoma (Wang et al., 1999), gastric cancer (Takeda et al., 2000), lung cancer (Wang et al., 1999), brain tumor (Nutt et al., 2000), oral carcinoma (Chang et al., 2000), nasopharyngeal carcinoma (Crook et al., 2000), esophageal carcinoma (Miyazaki et al., 2000), breast cancer (Pich et al., 2000), lymphoma (Boley et al., 2000), etc. In contrast, Wu et al. (2000) demonstrated that the sex steroids influence the growth of leiomyomas by stimulating cell proliferation rather than by affecting apoptosis. No difference in apoptotic index was observed between leiomyomas and normal myometrium (Wu et al., 2000). Growth modulation of leiomyomas by hormone deprivation may occur via mechanisms independent of apoptosis (Burroughs et al., 1997). Recently, Denschlag et al. (2005) demonstrated the higher prevalence of p53 codon 72*Pro allele and Pro/Pro genotype among Caucasian women with uterine leiomyoma. In contrast, in our previous survey, we observed the p53 codon 72 polymorphism was not associated with the susceptibility of leiomyoma in Asians (Hsieh et al., 2004).

**TABLE 3. The Allelic Frequency of Mutated Type of SNP in p53 Promoter Gene Between Women With and Without Endometriosis**

| Allele | Leiomyoma allele no. = 320 (%) | Nonleiomyoma allele no. = 400 (%) | *P* value |
|---|---|---|---|
| −250 A/G | 22 (6.9) | 15 (3.8) | 0.059 |
| −216 T/C | 16 (5.0) | 7 (1.8) | 0.014 |
| −103 A/G | 19 (5.9) | 9 (2.3) | 0.011 |
| −33 A/G | 12 (3.8) | 16 (4.0) | 0.86 |

The mutant p53 promoter could alter the p53 gene expression. The mutations might either increase or reduce promoter activity. The deregulated expressions of p53 promoter might play a role in the predisposition towards leiomyoma development. Point mutations in the DNA element of promoter regions might eliminate protein-DNA interactions as well as nonresponsive p53 genes. However, the involvement of p53 promoter mutation upon individual diseases remains anonymous to date. Furthermore, the role of p53 promoter in p53 expression is still elusive.

P53 promoter is related with the expression of p53 transcription. However, the involvement of p53 promoter in cancers is not clearly known. Few reports are available on mutations in the p53 promoter in cancers. Deletion analysis of p53 promoter delineated sequences between +22 and +67 as being critical for regulation (Deffie et al., 1993). Attwooll et al. (2002) identified a single nucleotide deletion within the C/EBPlike site of the promoter in human fibroblasts, which might be correlated with the susceptibility of Li-Fraumeni syndrome. They detected one silent gene polymorphism at codon 213 in exon 6 with CGA → CGG (Arg → Arg) change. They also observed that this mutation is not related with p53 production. Then they suggested that this site is not utilized in wild-type P53 promoter as well as its mutation has no consequent effect (Attwooll et al., 2002).

In contrast, Kullmann et al. (1999) analyzed the sequence p53 promoter, which did not reveal any mutational base change in the 10 synovial fibroblast

populations examined. This indicates that in these patients p53 mutations in synovial fibroblasts do not contribute to the proliferative and aggressive behavior of these cells. Furthermore, Nayak and Das (1999) demonstrated the absence of mutations and deletions in p53 promoter, which indicated that mutation of p53 promoter is probably not a significant factor in breast tumorigenesis. In fact, the specific mutation pattern of p53 gene appears different expression depending on the types of tissue (Kullmann et al., 1999).

In this survey, we sequenced the p53 promoter to determine whether promoter mutations could be responsible for the leiomyoma. We identified four novel SNPs within this region and observed that two of these mutated SNPs might be associated with the susceptibility of leiomyoma. It provided the evidence that these sequence variances might have a functional effect upon p53 presentation as well as leiomyoma development. These discrepancies with some previous reports might be due to ethologic variations, illness classification, and different sample origins.

In conclusion, in our preliminary survey, we identified as many as 15 mutation points within p53 promoter region. Among these mutations, a total of four SNPs might be established. We also observed that p53 promote −216*C and −103*G related genotypes and alleles might be associated with leiomyoma development. It suggested that sequence variations of p53 promoter might be useful markers for predicting leiomyoma susceptibility. These novel SNPs deserved further detailed disease-association studies to determine their linkage with individual diseases. However, correlation between SNPs in the p53 promoter region and leiomyoma susceptibility remains to be explored. Future studies need to determine if the polymorphisms in p53 promoter region have direct effect on the level of p53 transcription and ultimately tumor pathology.

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